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# Selection of antibodies for immunohistochemical detection of Bcl-2 family members in dogs and immunohistochemical expression pattern of Mcl-1 and Bcl-x in canine normal organs and lymphomas

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# List of abbreviations

| | |
|---|---|
| A1 | Bcl-2 related protein A1 |
| AEC | 3-amino-9-ethyl-carbazole |
| ANOVA | Analysis of variance |
| Apaf-1 | Apoptotic protease activating factor 1 |
| APS | Ammonium persulphate |
| Bad | Bcl-2 antagonist of death |
| Bak | Bcl-2 antagonist/killer |
| Bax | Bcl-2 associated X protein |
| Bcl-2 | B-cell CLL/lymphoma 2 |
| Bcl-w | Bcl-2 like 2 (BCL2L2) protein |
| Bcl-x | Bcl-2 like 1 (BCL2L1) protein |
| BH domain | Bcl-2 homology domain |
| Bid | BH3 interacting domain death antagonist |
| Bik | Bcl-2 interacting killer |
| Bim | Bcl-2 like 11 (BCL2L11) protein |
| DDT | Dichlordiphenyltrichlorethan |
| DNA | Desoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| GST | Glutathione-S-Transferase |
| HPA | Human Protein Atlas |
| HRP | Horseradish peroxidase |
| hu | Human |
| IHC | Immunohistochemistry |
| IPTG | Isopropyl-beta-D-thiogalactopyranoside |
| LB | Lysogeny broth |
| mAb | Monoclonal antibody |
| Mcl-1 | Myeloid cell leukaemia sequence 1 |
| $MgCl_2$ | Magnesium chloride |
| mo | Mouse |
| NaCl | Sodium chloride |
| Noxa | Phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1) |
| OD | Optical density |
| pAb | Polyclonal antibody |
| PBS | Phosphate-buffered saline |
| PMSF | Phenylmethylsulfonyl fluoride |
| Puma | Bcl-2 binding component 3 |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TBST | Tris-buffered saline containing Tween |
| TEMED | Tetramethylethylenediamine |
| TMA | Tissue microarray |
| UV(B) | Ultraviolet (B) |
| WB | Western blotting |

# 1. Summary

The Bcl-2 family plays an important role in tumourigenesis but its role in the genesis of canine lymphoma is yet unexplored. The aims of the present study were to select antibodies for immunohistochemical detection of various Bcl-2 family members in canine tissues and to analyse the expression of two anti-apoptotic members, Mcl-1 and Bcl-x, in normal canine and lymphoma tissues. A total of twelve antibodies out of a panel of twenty-one were deemed to specifically cross-react with canine recombinant Mcl-1, Bcl-x, Bcl-w, Bak and Bax expressed in bacteria. This was determined using an immunohistochemical method in formalin-fixed, paraffin-embedded bacteria and confirmatory Western blotting. Immunohistochemical protocols for Mcl-1 and Bcl-x were further optimized for use in canine tissues using cultured canine cells and selected normal tissues fixed in formalin and embedded in paraffin. Immunohistochemical expression patterns of these two proteins in normal canine tissues, detected using tissue arrays, are described here for the first time. They roughly coincide with the expression patterns reported in human tissues. With the same techniques, canine lymphomas were shown to frequently express Mcl-1 and, to a lesser extent, Bcl-x, across a wide range of different subtypes. These findings suggest a role for these two anti-apoptotic Bcl-2 family members in the pathogenesis of canine lymphoma.

# 2. Literature overview: The Bcl-2 protein family and its role in cancer with special reference to lymphoma

## 2.1. Introduction

Apoptosis is a form of programmed cell death with typical morphologic appearance, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr et al., 1972). It is executed by members of a family of specialized enzymes, called caspases, which digest proteins at specific cleavage sites (Kroemer et al., 2009).

Besides a number of other functions, one of the principal role of apoptosis is to contribute to the prevention of cancer by eliminating cells undergoing malignant transformation, e.g. as a consequence of genomic damage. Perturbances of this mechanism play therefore a key role in the development and maintenance of cancer (Ameisen, 1994) as well as in the development of therapeutic drug resistance (Hanahan and Weinberg, 2000; Johnstone et al., 2002).

Two main apoptotic pathways have been described. The extrinsic pathway is engaged when external ligands bind to specific receptors on the cell surface leading to the activation of caspase-8 and downstream caspases. The intrinsic or mitochondrial apoptotic pathway is triggered by situations of "cellular stress" including e.g. genomic damage and oncogene activation. Its activation results in permeabilization of the outer mitochondrial membrane, release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm (Cory and Adams, 2002) and assembling of the apoptosome, a multimolecular complex containing the adapter molecule Apaf-1 and mediating caspase-9 activation (Figure 1). Apoptosis ensues after cleavage and activation of further downstream caspases.

The Bcl-2 protein family plays a central role in the intrinsic pathway since it controls the integrity of the outer mitochondrial membrane. The following review outlines the principal characteristics of this protein family and details current knowledge on its role in the genesis and maintenance of lymphoma.

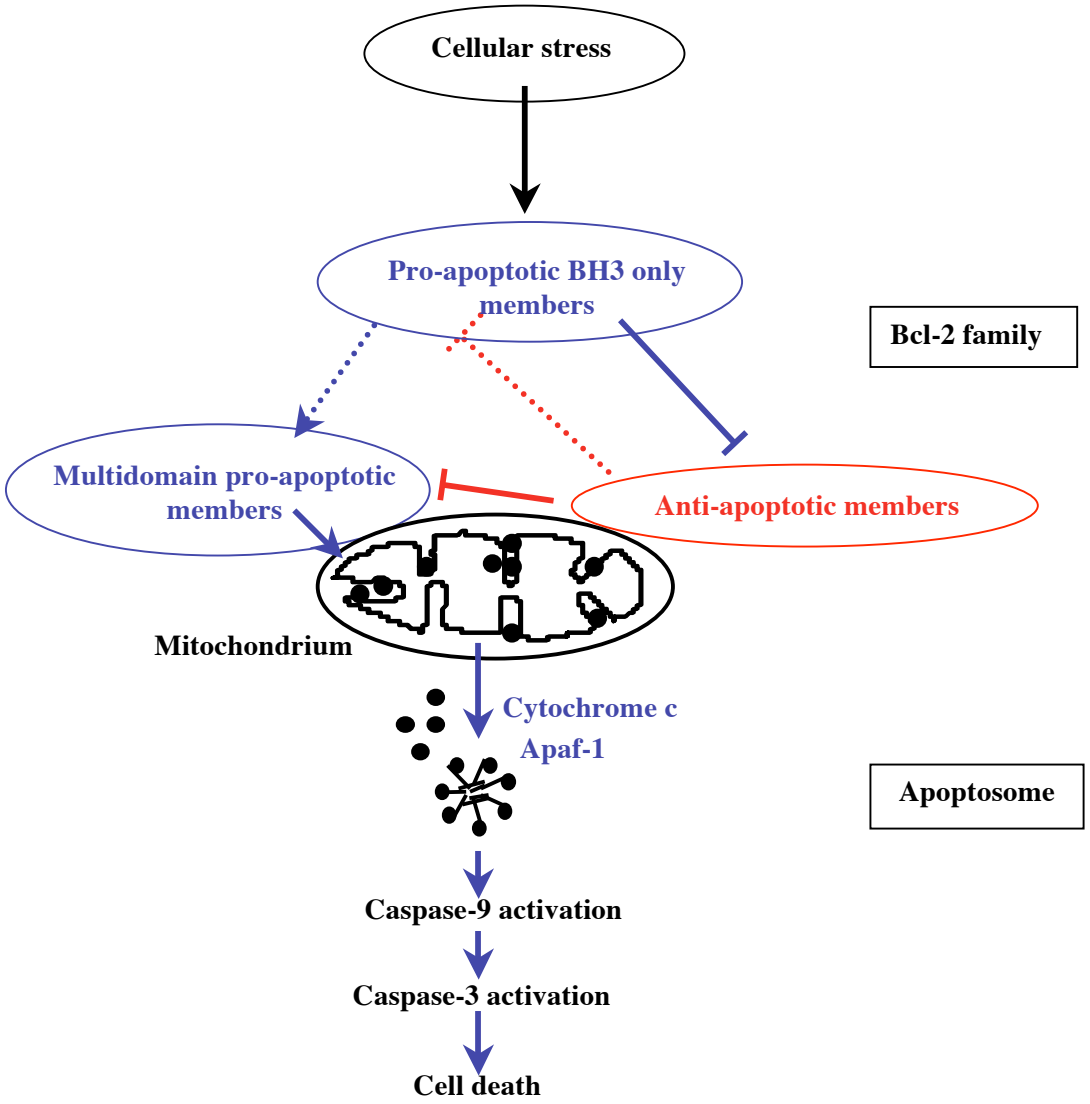



**Figure 1:** Simplified schematic depiction of the intrinsic apoptotic pathway and interactions of the Bcl-2 family members

activation
repression

## 2.2. The Bcl-2 protein family

B-cell CLL/lymphoma 2 (Bcl-2) was identified as the founder member and eponym of this protein family in human follicular lymphoma in 1984 (Tsujimoto et al., 1984). To date, almost twenty relatives are known in mammals and other eukaryotic organisms. The presence of at least one of four conserved Bcl-2 homology (BH) domains, which mediate interactions between the family members, is a prerequisite for inclusion of a protein in this family. These domains are strongly conserved in mammals and in lower organisms. In a previous study, where the sequences of several Bcl-2 family members were analysed, most of the BH domains were identical between humans and dogs (Schade, 2007).

The family consists of anti-apoptotic (pro-survival) and pro-apoptotic members. The anti-apoptotic members possess four BH domains and a transmembrane domain; they comprise the proteins Bcl-2, Bcl-$x_L$, Bcl-w, Mcl-1. As an exception, Mcl-1 lacks a BH4 domain. The pro-apoptotic members build two subfamilies. The multidomain pro-apoptotic members like the proteins Bak and Bax, display domains BH1 to BH3 and a transmembrane domain. These members are required for executing apoptosis through the mitochondrial pathway (Wei et al., 2001). Following a pro-apoptotic stimulus, they homo-oligomerize and build pores in the outer mitochondrial membrane allowing the efflux of pro-apoptotic factors from the mitochondrial intermembrane space into the cytoplasm. The other subfamily consists of proteins possessing only a BH3 domain, the BH3-only members. They are represented by Noxa, Bad, Bim, Puma and many others (Cory and Adams, 2002). The BH3 domain of BH3-only proteins mediates binding to and regulation of other family members (Youle and Strasser, 2008).

In the normal viable cell, it appears that the principal function of the anti-apoptotic members is to inhibit the activation of the multidomain pro-apoptotic members. The BH3-only members act as sensors of cellular stress and modulate the interaction between the other two subfamilies. The modalities of this modulation are currently subject of intense debate. The direct activation model (Figure 2A) divides the BH3-only proteins into a subgroup, which can bind only anti-apoptotic members and a second subgroup, which additionally can bind and activate the multidomain pro-apoptotic members (Deng et al., 2007; Letai, 2008). The former members (e.g. Bad, Noxa) are termed sensitizers since they can occupy binding sites on the anti-apoptotic proteins thereby provoking the release of BH3-only members of the latter subgroup (e.g. Bid, Bim and possibly Puma). These are thought to activate the multidomain pro-apoptotic members and are thus called activators. However, the activators bind to

multidomain pro-apoptotic proteins with a much lower affinity than they do to anti-apoptotic members (Chen et al., 2005). This model postulates the existence of further yet unknown activators unrelated to the Bcl-2 family (Letai, 2008). According to the second currently proposed model, the indirect activation model, only the binding of BH3-only proteins to anti-apoptotic proteins is physiologically relevant (Adams and Cory, 2007). Accordingly, the BH3-only proteins are further divided into promiscuous binders (i.e. such members capable of binding all anti-apoptotic molecules, e.g. Bid, Bim and Puma) and selective binders (i.e. such members able to bind only a subset of the anti-apoptotic proteins, e.g. Noxa and Puma, which bind Mcl-1 and A1 or Bcl-2, Bcl-x and Bcl-w, respectively) (Figure 2B). The promiscuous binders are more potent in inducing apoptosis than the selectively binding BH3-only proteins (Adams and Cory, 2007; Youle and Strasser, 2008), but different selective binders potently induce cell death if combined (Chen et al., 2005).

Regardless of which of these models might reveal to be correct, the picture emerges of a strong redundancy in this part of the apoptotic pathway, although diverse non-redundant roles have been attributed to some proteins of the family under physiological conditions. These include, for instance, a critical role for anti-apoptotic Mcl-1 in lymphoid development and maintenance of mature lymphocytes (Opferman et al., 2003). Another important protein in this respect is the BH3-only member Bim, which provokes apoptosis of lymphocytes following selected cytotoxic stimuli. It is implicated in the elimination of thymocytes that recognize self-antigens and is critical for terminating T-cell immune responses (Adams and Cory, 2007).

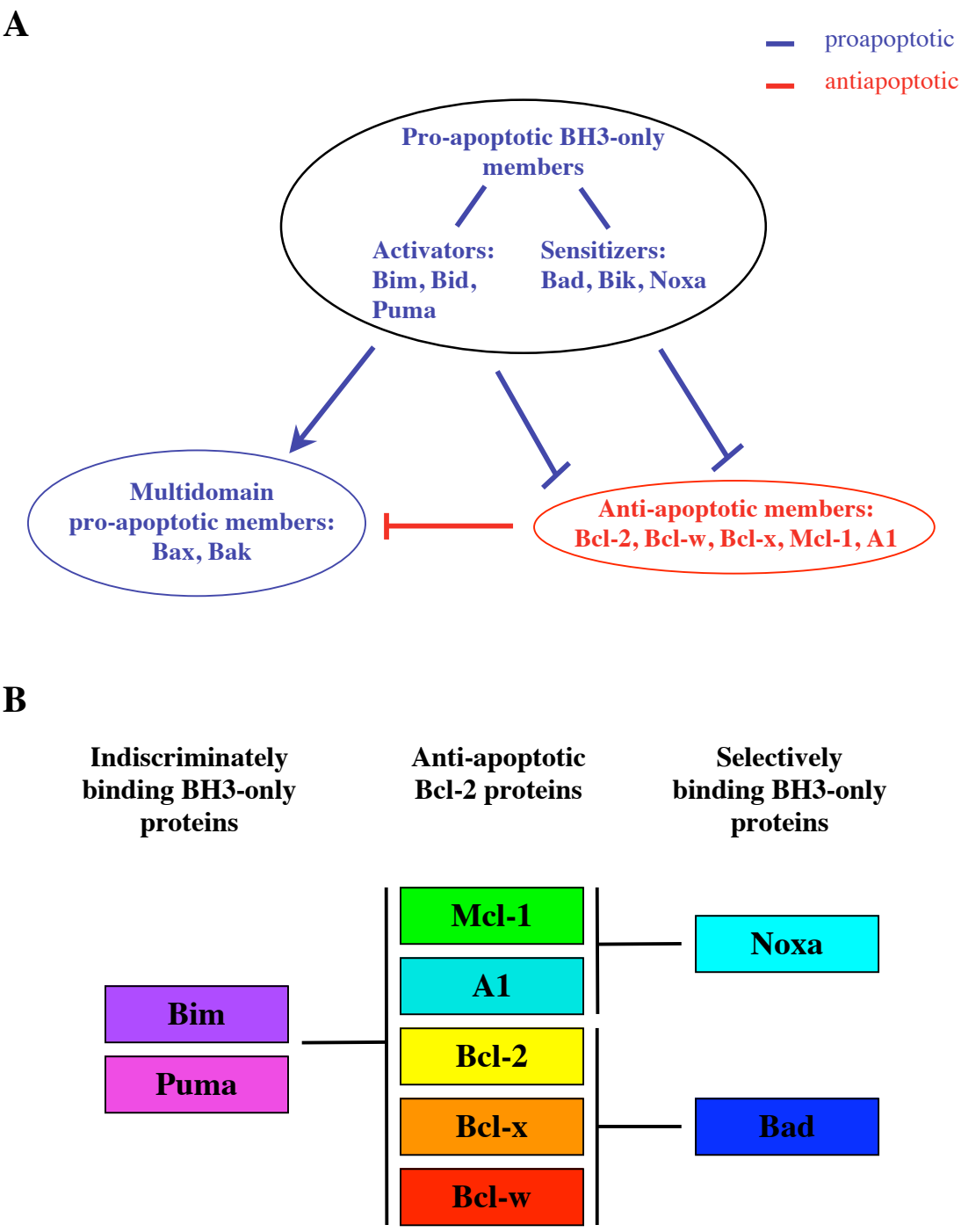



**Figure 2:** Current models of the interactions of selected Bcl-2 family proteins
A. Interactions according to the direct activation model; B. Interaction types according to the indirect activation model and based on binding specificities of selected BH3-only proteins to anti-apoptotic Bcl-2 family members (modified from Adams and Cory, 2007).

## 2.3. Bcl-2 family and lymphoma/lymphatic leukaemia

Animal models have provided a frame to study the oncogenic effect of anti-apoptotic Bcl-2 family proteins and have contributed to elucidate the roles of various members in diverse tumour types. Lymphomas are frequently involved, or specifically addressed, in such models. The role of anti-apoptotic molecules has been addressed in the transgenic setting. For instance, the E*μ-bcl-2* transgenic mouse, an early model consisting of a B-lymphoid-specific transgene mimicking the translocation observed in human follicular lymphomas, confirmed the oncogenic effect of deregulated Bcl-2 expression (McDonnell and Korsmeyer, 1991). A low incidence of tumours, mostly plasmacytomas, and a long latency were observed in these animals, and in most neoplasms translocations of the *myc* gene occurred (Strasser et al., 1993). Another transgene model with pan-hematopoietic Bcl-2 expression preferentially developed follicular lymphoma (Egle et al., 2004). A synergy between Myc and Bcl-2 was confirmed by findings in the E*μ-bcl-2*/ E*μ-myc* bitransgenic mouse, since in these animals development of tumours was accelerated as compared to the transgenes with either gene alone (Strasser et al., 1990). It was further shown that Myc overexpression enhances the expression of Bim and reduces the expression of Bcl-$x_L$ and Bcl-2. Mice transgenic for Mcl-1 developed a variety of different B-cell lymphoma subtypes, which appeared after long latency and with high probability (Zhou et al., 2001). Mice transgenic for B-cell restricted Bcl-$x_L$ and c-myc developed acute, aggressive lymphoblastic leukaemia, while the transgenes overexpressing only Bcl-$x_L$ developed large expansions of pro-B cells in bone marrow but no neoplasia (Fang et al., 1996; Swanson et al., 2004). Animal models to investigate the function of pro-apototic Bcl-2 family proteins are based in large part on knock-out technology. Development of cancer after loss of a gene would be supportive of a protective role for this gene in tumourigenesis. Likely because of the strong degree of redundancy in this gene family, loss of a single pro-apoptotic member is usually not associated with changes in the phenotype or is associated with minimal changes. Loss of Bax and Bak provoked high perinatal mortality. Double knock-outs surviving to adult age showed extensive accumulation of lymphoid and myeloid cells (Lindsten et al., 2000). Moreover, loss of Bax accelerated development of Myc-induced lymphomas indicating a protective role for Bax (Eischen et al., 2001). Similarly, loss of Bim, even monoallelic, accelerated the development of acute B-cell leukaemia in E*μ-myc* transgenes (Egle et al., 2004). Despite their minimal phenotype in young age, Bad-deficient mice developed, with aging, diffuse large B-cell lymphomas of germinal centre origin. In addition, exposure of Bad-null mice to sublethal gamma-irradiation resulted in an increased

incidence of pre-T cell and pro-/pre-B cell lymphoblastic leukaemia/lymphoma (Ranger et al., 2003). In conclusion, the animal models discussed above support a role for several Bcl-2 family proteins in the genesis of tumours of lymphatic origin.

Proponents of the direct activation model have postulated the occurrence in cancer cells of three different possible blocks of apoptosis in the intrinsic pathway at the level of the Bcl-2 family. Accordingly, a class A block derives from a loss of expression or a loss of function of activators, class B blocks ensue from defects of the multidomain pro-apoptotic proteins and in class C blocks the pro-apoptotic components are unaltered while anti-apoptotic proteins are variably overexpressed (Deng et al., 2007). It appears from literature that abnormal expression of relevant Bcl-2 family members is a frequent cause of the blocks described above in lymphoma (and other cancer types) in humans, although several other mechanisms have been described as well. Enhanced expression is expected to be important for anti-apoptotic proteins. Besides the t(14;18) chromosomal translocation observed in human follicular lymphoma mentioned in the preceding chapter, gene amplification and loss of endogenous microRNAs have been reported to be associated with overexpression of Bcl-2 (Tsujimoto et al., 1984; Rantanen et al., 2001; Calin et al., 2008). The latter mechanism has also been shown to lead to overexpression of Mcl-1 (Calin et al., 2008). Alteration of signalling pathways is common in neoplastic cells and this can result in altered transcriptional regulation of genes involved in apoptosis. For example, in malignant lymphocytes transcription of Bcl-x was recently reported to be driven from additional elements other than those responsible for basal transcription (Habens et al., 2007). Conversely, loss of expression may be expected to commonly occur with anti-apoptotic proteins. Indeed, loss of Bax expression has been reported in tumour cell lines including acute lymphatic leukaemia cells (Meijerink et al., 1998). A more important and frequent cause of diminished expression in malignant cells is probably the loss of p53 function, a very common event in cancer, which may negatively influence at the transcriptional level the expression of target genes such as Bax, Bid, Puma and Noxa (Yip and Reed, 2008). Biallelic deletion of the *Bim* gene or silencing through promotor hypermethylation was detected in mantle cell and Burkitt lymphomas, respectively (Tagawa et al., 2005; Mestre-Escorihuela et al., 2007). Notably, the latter lymphoma subtype is characterized by Myc translocation. For Bim, enhanced degradation through the proteasome constitute an additional, post-translational mechanism regulating the amount of protein (Adams and Cory, 2007). Some other BH3-only members, however, are maintained in the cytoplasm in an inactive form and they are activated through further post-translational modifications. For instance, activation of Bad requires

a dephosphorylation step provoking its release from 14-3-3 scaffold proteins. In such cases, detection methods measuring the amount of protein in the cell may be misleading, since the protein present may not be functional. In the case of Bad, it may be necessary to determine the level of phosphorylated protein as well. In one study, a high level of phosphorylated Bad was demonstrated in a cutaneous T-cell lymphoma line and it was suspected to be responsible for the resistance to therapy observed (Zhang et al., 2003).

Missense mutations can elicit changes in the activity of a protein. In general, however, mutations in the genes coding for the core apoptotic machinery appear to be relatively infrequent to rare (Packham, 1998). Mutations of the Bax gene, resulting partly in loss of immunodetectable Bax and partly in substitutions of single amino acids, were found in approximately 21% of hematopoietic cell lines and perhaps most commonly in acute lymphatic leukaemia (Meijerink et al., 1998). Another study, however, found no such mutations in clinical cases of this tumour type (Prokop et al., 2000). In some cases of lymphoma, missense mutations have been described in the Bcl-2 gene and they have been proposed to be related to its translocation to a hypermutation-prone environment (Packham, 1998). In one study, a missense mutation was reported in one out of fifty diffuse large B-cell lymphomas analysed (Yamaguchi et al., 2002). Another study has reported mutations of the gene coding for Noxa in a few human lymphomas (Mestre-Escorihuela et al., 2007).

Due to the prominence in tumourigenesis of abnormalities related to the amount of Bcl-2 family proteins expressed, investigations relying on their measurement such as immunohistochemistry have been widely used to characterise a wide range of cancer types, including lymphomas in humans. Preferential expression of some Bcl-2 family genes has been linked to certain lymphoma subtypes in some cases (Agarwal and Naresh, 2002; Rassidakis et al., 2003) and in several instances overexpression was correlated either with the proliferation index or the apoptotic rate, or with prognosis. For example, Bcl-2 (over)expression has repeatedly been correlated with unfavourable outcome (Adams and Cory, 2007). Expression of Bcl-x, detected by RT-PCR, was shown to be of negative prognostic significance in follicular lymphoma (Zhao et al., 2004). Some studies suggest, based on clinical findings, that cooperation of various anti-apoptotic molecules might be responsible for the pathogenesis of lymphomas (Ghia et al., 1998; Bairey et al., 1999; Zhang et al., 2003). Sometimes, combinations of expressed amounts of different members have been proposed to be of prognostic significance. For instance, high ratios of Bcl-2/Bak and Bcl-2/Bax identified using a proteomics approach have been shown to be associated with early death from disease in human follicular lymphoma (Gulmann et al., 2005). In some

cases, somehow paradoxical results were reported, such as association of high expression of Bax, detected by Western blotting, with high risk of relapse in childhood acute lymphatic leukaemia (Hogarth and Hall, 1999). It has been hypothesized that synthesis of non-functional Bax might be responsible for resistance to therapy in cutaneous T-cell lymphomas (Zhang et al., 2003). Conversely, it has been shown that both, Bax expression levels and the Bax/Bcl-2 ratio, are significantly lower in samples at relapse than in samples at initial diagnosis in childhood acute lymphatic leukaemia (Prokop et al., 2000).

Cytotoxic stimuli elicited by traditional anticancer drugs mostly rely on Bcl-2 family-dependent apoptotic mechanisms for the demise of tumour cells. Since in many tumour types the core apoptotic machinery is intact, several therapeutic approaches targeting anti-apoptotic Bcl-2 family members have demonstrated potential efficacy in preclinical models including lymphoid neoplasia. For example, antisense treatment against Bcl-x has been shown to sensitize T-cell acute leukaemia cells to chemotherapy drugs (Broome et al., 2002). Gossypol, a non-peptidic small-molecular inhibitor of Bcl-2 and Bcl-x, showed anti-tumour activity against diffuse large B-cell lymphoma cells, either alone or in combination with standard chemotherapy (Mohammad et al., 2005). The apoptotic response following therapy often is triggered through sensing by specific BH3-only proteins (Adams and Cory, 2007). This renders the use of therapeutic modalities relying on BH3 mimetics attractive, since specific BH3 mimetics are likely to be usable against a subset of tumours overexpressing the corresponding proteins, resulting in reduced toxicity for normal tissues. For instance, the drug ABT-737 was shown to act as a Bad-mimetic and to have an anti-tumoural effect in preclinical tests and in a murine myeloid leukaemia model (Konopleva et al., 2006). Resistance to this drug was due either to lack of both Bax and Bak or to enhanced expression of Mcl-1 (Konopleva et al., 2006).

In conclusion, alterations of members of the Bcl-2 family of proteins are a common finding in neoplasias of the lymphatic tissues and they contribute to their genesis, maintenance and resistance to therapy. These alterations frequently, albeit not exclusively, consist of changes in the level of expression of the proteins, thus suggesting that methods assessing these parameters, such as immunohistochemistry, are an appropriate tool to investigate their involvement.

Non-Hodgkin lymphoma is the most frequently occurring neoplasm in dogs. It has been reported to account for 90% of hematopoietic tumours in this species and to have an incidence rate of 13 to 24 per 100 000 dogs (Dorn et al., 1967). Other studies have reported similar estimates, with an minimum incidence rate of 33 per 100 000 dogs in the Netherlands (Teske,

1994) respectively of 134 per 100 000 cases of lymphoid malignancies per year in the UK (Dobson et al., 2002). Due to the importance of lymphoma for the dog and to the similarities of the canine disease with the human counterpart and its potential as a model, studies of aspects related to alteration of apoptosis are in this tumour necessary. To the knowledge of the author, there are no data about the role of Bcl-2 family proteins in canine lymphoma available yet.

# 3. Aim of the study

The present work deals with two tasks. The first aim was to advance a previously described test system (Keller et al., 2007) for selection of commercially available anti-human and/or anti-mouse antibodies for further use in the immunohistochemical assay of canine tissues. For this purpose, a panel of antibodies against Bcl-2 family proteins Mcl-1, Bcl-x, Bcl-w, Bak, Bax, Noxa and Bad, was tested on bacterially-expressed, recombinant canine proteins by an immunoperoxidase method. Western blotting was used to confirm parts of the results obtained by the immunohistochemical test system. The second aim was to determine the expression patterns of the anti-apoptotic proteins Mcl-1 and Bcl-x in normal canine tissues and in various canine lymphoma samples. For this purpose, immunohistochemical protocols, based on two specific cross-reacting antibodies selected in the first part of the study, were adapted for immunohistochemistry of canine tissues. The protocols were established using cultured canine keratinocytes, as well as normal canine skin and lymph nodes. The immunohistochemical expression patterns for these two proteins were examined in two sets of canine tissues assembled into tissue microarrays. One set consisted of normal organs and the second comprised ninety-three archival lymphoma samples previously classified.

# 4. Materials and methods

## 4.1. Primary antibodies against Bcl-2 family proteins

All antibodies used in this study are commercially available and were raised against human and/or murine epitopes. A summary of their main characteristics is given in Table 1. Based on the availability of information, the selection criteria were a high degree of canine-to-human or canine-to-murine sequence homology of the immunogen used to generate the antibody and affinity-purification of the antibody. In addition, as far as possible, antibodies against different epitopes were chosen.

***Antibodies against Mcl-1***

1. Anti-human Mcl-1 (Clone RC13) mouse monoclonal antibody, catalog no. MAB4602, manufactured for Chemicon International by Millipore Headquarters, 290 Concord Road, Billerica, MA 01821, USA.
2. Anti-human Mcl-1 affinity-purified rabbit polyclonal antibody, catalog no. HPA008455-100UL, Lot no. R02217, manufactured by Atlas Antibodies AB, AlbaNova University Center, SE-106 91 Stockholm, Sweden.
3. Anti-human Mcl-1 rabbit polyclonal antibody, catalog no. A3534, Lot no. 057, manufactured by Dako Corporation, 6392 Via Real, Carpinteria, CA 93013, USA.
4. Anti-human Mcl-1 rabbit polyclonal antibody, catalog no. PA1-21067, Lot no. 15628, manufactured by ABR Affinity BioReagents, 4620 Technology Drive, Suite 600, Golden, CO 80403, USA.

***Antibodies against Bcl-x***

1. Anti-human Bcl-x Ab-1 (Clone 2H12) mouse monoclonal antibody, catalog no. MS-715-PO, manufactured by NeoMarkers for Lab Vision Corporation, Thermo Fisher Scientific, Anatomical Pathology, 47777 Warm Springs Blvd., Fremont, CA 94539, USA.
2. Anti-mouse Bcl-$x_L$ Ab-2 (Clone 7D9) mouse monoclonal antibody, catalog no. MS-1334-PO, manufactured by NeoMarkers for Lab Vision Corporation, Thermo Fisher Scientific, Anatomical Pathology, 47777 Warm Springs Blvd., Fremont, CA 94539, USA.

3. Anti-human Bcl-x polyclonal rabbit antibody, catalog no. RB-9205-PO, Lot no. 9205P401, manufactured by Lab Vision Corporation, 47790 Westinghouse Dr. Fremont, CA 94539, USA.

***Antibodies against Bcl-w***

1. Anti-human/mouse Bcl-w affinity-purified rabbit polyclonal antibody, catalog no. AF824, Lot no. BVZ04, manufactured by R&D Systems Inc., 614 McKinley Place NE, Minneapolis, MN 55413, USA.
2. Anti-human Bcl-w (16-29) immunoaffinity purified rabbit polyclonal antibody, catalog no. 197209, Lot no. D00034231, manufactured by Calbiochem®, Merck Chemicals Ltd., 10394 Pacific Center Court, San Diego, CA 92121, USA.
3. Anti-Bcl-w (Clone 6C1) mouse monoclonal antibody, catalog no. NCL-Bcl-w, manufactured by Novocastra Laboratories Ltd., Balliol Business Park West, Benton Lane, Newcastle upon Tyne NE12 8EW, United Kingdom.

***Antibodies against Bak***

1. Anti-human Bak rabbit monoclonal antibody (Clone Y164), catalog no. 1542-1, manufactured by Epitomics Inc., 863 Mitten Road, Suite 103 Burlingame, CA 94010-1303, USA.
2. Anti-human Bak rabbit polyclonal antibody, catalog no. 06-535, Lot no. 31936, manufactured by Upstate, Cell Signaling Solutions, 48 Barn Road, Lake Placid, NY 12946, USA.
3. Anti-mouse Bak (Ab-2) monoclonal mouse antibody, catalog no. AM04, manufactured by Calbiochem®, Merck Chemicals Ltd., 10394 Pacific Center Court, San Diego, CA 92121, USA.

***Antibodies against Bax***

1. Anti-mouse Bax (B-9) mouse monoclonal antibody, catalog no. sc-7480, manufactured by Santa Cruz Biotechnology Inc., 2145 Delaware Avenue, Santa Cruz, CA 95060, USA.
2. Anti-human Bax rabbit polyclonal antibody, catalog no. 06-499, Lot no. 24156, manufactured by Upstate, Cell Signaling Solutions, 48 Barn Road, Lake Placid, NY 12946, USA.

3. Anti-human Bax (Ab-1) rabbit polyclonal antibody, catalog no. PC66T, Lot no. D00002969, manufactured by Oncogen, Merck Chemicals Ltd., 10394 Pacific Center Court, San Diego, CA 92121, USA.
4. Anti-human Bax (A20) rabbit polyclonal antibody, catalog no. DB005, Lot no. G1007, manufactured by Delta Biolabs, LLC 8870 Muraoka Drive, Gilroy, CA 95020, USA.
5. Anti-human Bax (Apoptosis Marker) Ab-1 (Clone 2D2) mouse monoclonal antibody, catalog no. MS-711-P0, manufactured by NeoMarkers for Lab Vision Corporation, Thermo Fisher Scientific, Anatomical Pathology, 47777 Warm Springs Blvd., Fremont, CA 94539, USA.

***Antibodies against Noxa***

1. Anti-mouse Noxa/Phorbol-12-myristate-13-acetate-induced Protein 1 (PMAIP1) rabbit polyclonal antibody, catalog no. LS-B184, Lot no. 072402A, manufactured by LifeSpan Biosciences Inc., 2401 Fourth Avenue, Suite 900, Seattle, WA 98121, USA.
2. Anti-mouse Noxa (PMA-induced protein 1, PMAIP1, APR) rabbit polyclonal antibody, catalog no. 54076, Lot no. HE093, manufactured by AnaSpec Inc., 2149 O'Toole Avenue, San Jose, CA 95131, USA.

***Antibodies against Bad***

1. Anti-human Bad (C-7) mouse monoclonal antibody, catalog no. sc-8044, manufactured by Santa Cruz Biotechnology Inc., 2145 Delaware Avenue, Santa Cruz, CA 95060, USA.

**Table 1:** Primary antibodies against Bcl-2 family proteins used in this study

| Antibody[°] | Antibody type[*] | Immunogen[†] | Antibody concentration | Manufacturer/ Vendor |
|---|---|---|---|---|
| **Mcl-1 no. 1** | mAb | aa 1-327 hu | 0.4 µg/µl | Chemicon |
| **Mcl-1 no. 2** | pAb | aa 117-129 hu | 0.2 µg/µl | Atlas Antibodies |
| **Mcl-1 no. 3** | pAb | aa 121-139 hu | 1 µg/µl | Dako Corporation |
| **Mcl-1 no. 4** | pAb | aa 121-139 hu | 0.002 µg/µl | ABR Affinity BioReagents |
| **Bcl-x no. 1** | mAb | aa 3-14 hu | 0.2 µg/µl | NeoMarkers |
| **Bcl-x no. 2** | mAb | aa 35-50 mo | 0.2 µg/µl | NeoMarkers |
| **Bcl-x no. 3** | pAb | aa 46-66 hu | 0.2 µg/µl | Lab Vision |
| **Bcl-w no. 1** | pAb | aa 32-51 hu and mo | 1.2 µg/µl | R&D Systems |
| **Bcl-w no. 2** | pAb | aa 16-29 hu | 1 µg/µl | Calbiochem |
| **Bcl-w no. 3** | mAb | full-length protein excluding the BH4 domain hu | 0.024 µg/µl | Novocastra |
| **Bak no. 1** | mAb | residues near the N-terminus hu | 0.125 µg/µl | Epitomics |
| **Bak no. 2** | pAb | aa 23-38 hu | 1 µg/µl | Upstate |
| **Bak no. 3** | mAb | full-length protein excluding the C-terminus and transmembrane domain hu | 0.1 µg/µl | Calbiochem |
| **Bax no. 1** | mAb | aa 1-171 mo | 0.2 µg/µl | Santa Cruz |
| **Bax no. 2** | pAb | aa 1-21 hu | 0.87 µg/µl | Upstate |
| **Bax no. 3** | pAb | aa 150-165 hu | 0.1 µg/µl | Oncogene |
| **Bax no. 4** | pAb | aa 11-30 hu | 0.2 µg/µl | Delta Biolabs |
| **Bax no. 5** | mAb | aa 3-16 hu | 0.2 µg/µl | NeoMarkers |
| **Noxa no. 1** | pAb | aa 51-66 and aa 75-90 mo | 0.5 µg/µl | LifeSpan |
| **Noxa no. 2** | pAb | 17 aa at the N-terminus mo | 1 µg/µl | AnaSpec |
| **Bad no. 1** | mAb | aa 1-168 hu | 0.2 µg/µl | Santa Cruz |

[°] numbering of antibodies used in this study

[*] mAb=monoclonal antibody; pAb=polyclonal antibody. All mAbs are of mouse origin with exception of antibody Bak no. 1, which is of rabbit origin. All pAbs are of rabbit origin.

[†] hu=human protein sequence; mo=mouse protein sequence

### 4.2. Bacterial expression of recombinant canine Bcl-2 family proteins

Bacterial expression plasmids pGEX-4T2 (Invitrogen, Carlsbad, USA), containing the coding sequences for the canine Bcl-2 family members Mcl-1, Bcl-x, Bcl-w, Bak, Bax, Noxa and Bad in frame after a sequence coding for Glutathione-S-Transferase (GST), as well as the empty vector, were available from a previous study (Schade, 2007). The plasmids were transformed into BL21 Star™ chemically competent Escherichia coli (Invitrogen, Carlsbad, USA) for high-level protein expression with the following procedure. BL21 cells were removed from the freezer (-80 °C), directly placed on ice and thawed for 5 to 10 min. Between 1 to 50 ng of DNA was added into 50 µl competent cells and gently mixed by tapping. The samples were held on ice for 30 min. Subsequently, the cells were heat-shocked at 42 °C for 45 sec. Then, 450 µl of sterile lysogeny broth (LB) medium (Oxoid AG, Pratteln, Switzerland) at room temperature (RT) was added to each transformation reaction and incubated for 1 h at 37 °C. Finally, 150 µl of each reaction was plated on LB plates, supplemented with 50 µg/ml ampicillin (Sigma-Aldrich, Buchs, Switzerland) and all plates were incubated over night at 37 °C and stored at 4 °C, thereafter.

For expression experiments, one bacterial colony was picked from the corresponding plate and grown by shaking over night at 37 °C in 3 ml of LB medium supplemented with 50 µg/ml ampicillin. The saturated cultures were added to 500 ml cultures of sterile LB medium, supplemented with 50 µg/ml ampicillin and grown at 37 °C for approximately 3 h to an $OD_{600}$ of 0.5 – 0.6. At this point, protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, Buchs, Switzerland) to a final 1mM concentration. The bacteria were harvested after 2.5 h of further growth under the same conditions and were further processed either for paraffin wax embedding (whole bacteria), or for Western blotting or antibody preincubation experiments (protein extracts).

### 4.3. Western blotting (WB)

After protein induction, bacteria expressing the Mcl-1, Bcl-x, Noxa and Bad GST-fusion proteins, as well as GST alone, were pelleted by centrifugation of 1 ml culture volumes for 1 min at 13 000 rpm in an Eppendorf centrifuge at RT. The supernatant was discarded and the pellets were resuspended in 200 µl of 2x Lämmli-SDS-PAGE buffer containing 80 mM Tris/HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 10% glycerol, 0.004% bromphenol blue. After incubation for 3 min at 95 °C and centrifugation for 1 min at 13 000 rpm in an Eppendorf centrifuge at RT, the bacterial lysates were kept on ice and

loaded on 15% SDS polyacrylamide gels, containing 5 ml 4x lower buffer (1.5 M Tris/HCl pH 8.8, 0.4% SDS), 7.5 ml 40% acrylamide, 7.32 ml sterile water, 150 µl 25% ammonium persulphate (APS), 30 µl tetramethylethylenediamine (TEMED)) for separation by SDS-PAGE electrophoresis. The SDS-PAGE gel separated proteins were transferred to a nitrocellulose membrane by means of a semi-dry blotting system (Biometra, Göttingen, Germany), according to the instructions of the manufacturer. For blot analyses monoclonal or polyclonal antibodies against Bcl-2 family proteins (Table 2) or a polyclonal, horseradish peroxidase (HRP) conjugated goat anti-GST antibody (GE Healthcare, Amersham Biosciences Europe GmbH, Otelfingen, Switzerland) were used. Before application on membrane, monoclonal antibodies and the anti-GST-HRP antibody were diluted in Tris-buffered saline containing 0.05% Tween 20 (TBST, Dako Wash Buffer, Dako Schweiz AG, Baar, Switzerland) and 1% bovine serum albumin (BSA, Sigma-Aldrich, Buchs, Switzerland), while polyclonal antibodies were diluted in TBST containing 1% skimmed milk powder (Fluka, Sigma-Aldrich, Buchs, Switzerland). Unspecific antibody binding sites were blocked for 1h at RT either in TBST supplemented with 3% BSA for monoclonal antibodies or with TBST and 3% skimmed milk powder for polyclonal antibodies. After membrane wash for 3 times 15 sec and 2 times 10 min in TBST, the membrane was incubated for 2 hrs at RT, or over night at 4 °C, with primary antibodies against Bcl-2 family members (Table 2) or the anti-GST-HRP antibody. Excess of primary antibody was removed by 3 times 15 sec and 2 times 10 min wash with TBST. Afterwards secondary antibodies were applied for 1h at RT: either a goat-anti-mouse-HRP (Geno Technologies, St Louis, MO, USA) or a goat-anti-rabbit-HRP (Jackson ImmunoResearch, Soham, UK), both diluted 1 in 7500 in TBST containing 1% skimmed milk powder. After a further washing step as described with primary antibody, reactions were visualized by a chemiluminescence detection system. Membranes were incubated with LumiGlo substrate (KPL, Maryland, USA) for 1 min, covered with transparent foil and exposed for different periods of time to film (Hyperfilm™ ECL, Amersham, UK).

In some instances membrane was reused to derive extra information and bound antibodies were removed before blocking of unspecific antibody binding sites and incubation with further antibodies as needed. For this purpose, the membrane was immersed in stripping buffer containing 2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris/HCl pH 6.8 for 30 min at 50 °C. Washing steps in TBST were as described above.

**Table 2:** Primary antibodies against Bcl-2 family proteins used for Western blotting (WB)

| Antibody | Concentration for WB |
|---|---|
| Mcl-1 no. 1 | 1 µg/ml |
| Mcl-1 no. 2 | 0.78 mg/ml |
| Bcl-x no. 2 | 0.4 µg/ml |
| Noxa no. 1 | 1 µg/ml |
| Noxa no. 2 | 1 µg/ml |

## 4.4. Cell culture, ultraviolet B (UVB) irradiation, fixation and embedding of keratinocytes

Normal canine keratinocyte cultures (passage 18) (Kolly et al., 2005) were grown at 37 °C and a 5% $CO_2$ atmosphere in Dulbeccos' Modified Eagles medium (Gibco BRL Life Sciences, Basel, Switzerland), supplemented with 1% non-essential amino acids (Gibco BRL Life Sciences, Basel, Switzerland), 1% sodium pyruvate (Gibco BRL Life Sciences, Basel, Switzerland), 1% Penicillin-Streptomycin (Gibco BRL Life Sciences, Basel, Switzerland) and 15% fetal calf serum (Gibco BRL Life Sciences, Basel, Switzerland). The cells were plated and allowed to grow for 24 h until approximately 50% confluence was reached. Then, the culture medium was replaced with phosphate-buffered saline (PBS, Gibco, Invitrogen, Carlsbad, USA). To induce apoptosis, plates were irradiated at a dose of 100 mJ/cm$^2$ UVB using a DNA crosslinker (ltf Labortechnik, Wasserburg, Germany) with an emission peak at 312 nm. Four plates were irradiated at the same point in time with a single dose. Thereafter, the cells were further incubated in culture medium and harvested at 0 h, 6 h, 12 h and 24 h after irradiation. One additional plate, not exposed to UV-irradiation, but substituted with PBS during the irradiation of the other plates and harvested at 24 h, served as a negative control. For harvest, the medium was aspirated and cells were fixed in 4% neutral buffered formaldehyde for 1 h at RT. Thereafter, the cells were scraped off with a cell scraper and washed with PBS supplemented with two drops of Tween (Fluka, Sigma-Aldrich, Buchs, Switzerland). After centrifugation for 5 min at 3000g at RT and two washes with PBS, the cells were resuspended in 1.5 ml of 5% BSA in PBS and one drop of hemalum (Merck, Darmstadt, Germany) was added. Then, the mixture was centrifuged twice for 5 min at 3000g at RT and the supernatant was discarded. The resulting cell pellets were embedded in paraffin wax using the Cytoblock system (Thermo Shandon, Pittsburgh, PA, USA) and a routine embedding procedure.

### 4.5. Fixation and embedding of bacteria expressing recombinant GST-tagged canine Bcl-2 family members

Bacterial cultures (500 ml) induced to express GST-fusion proteins of the Bcl-2 family members Mcl-1, Bcl-x, Bcl-w, Bak, Bax, Noxa and Bad were centrifuged for 10 min at 4000g at 4 °C. The supernatant was discarded and the pellets were resuspended in 4% neutral buffered formaldehyde and incubated over night at RT. Subsequently, the cultures were centrifuged for 10 min at 4000g at RT, the pellets were resuspended in 1.5 ml of 5% BSA in PBS and two drops of hemalum were added. After centrifugation for 10 min at 13 000 rpm in an Eppendorf centrifuge at RT, the pellets were embedded in paraffin wax using the Cytoblock system as described above.

### 4.6. Tissue microarray (TMA) construction

Several arrays, as indicated below, were constructed using paraffin-embedded cells or tissues by means of a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Cylinders were collected from representative areas of each specimen and transferred to paraffin wax recipient blocks (Micro-Cut Paraffin, Polysciences Inc., Warrington, PA, USA).
The arrays were assembled and designated as follows:

1. ***Bacterial pellets array:*** this block included one core (diameter 1.2 mm) each of formalin-fixed, paraffin-embedded bacterial pellets expressing recombinant canine Mcl-1, Bcl-x, Bcl-w, Bak, Bax, Noxa, Bad GST-fusion proteins as well as GST alone.
2. ***Cultured canine keratinocytes array***: this block comprised 1.2 mm cores of each UV-irradiated cell pellet harvested at different points in time (0 h, 6 h, 12 h, 24 h after irradiation) and of the non-irradiated cell pellet.
3. ***Normal canine tissues arrays:*** three blocks contained 1.2 mm cores of all major organs, each derived from different dogs and free of histopathologic lesions. In total, tissues were collected from eight dogs. Specimens were taken from the dogs maximally 24 h after euthanasia and were fixed for 24 to 48 h in 4% neutral buffered formaldehyde before embedding in paraffin wax using routine methods. Some dogs were healthy and some were patients, whose tissue samples used for this study were free of histopathologic lesions. The number of available cores per organ varied from three to eight. The normal tissue samples originated from the skin, digestive tract (salivary glands, stomach, intestine, liver, pancreas), respiratory tract (trachea, bronchi, alveoli), cardiovascular system (heart), reproductive organs (ovary, uterus,

vagina, mammary gland, prostate, testis), musculoskeletal system (skeletal muscle), hematopoietic/lymphoid system (spleen, tonsil/lymph node, thymus, bone marrow), urinary tract (kidney, bladder), endocrine organ (thyroid) and central nervous system (brain, spinal cord).

4. ***Canine lymphoma arrays***: three blocks included ninety-three canine malignant lymphoma samples derived from archival material (from the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich) and consisted of four cores of 0.6 mm in diameter taken from each tumour sample. Part of the array material (40 cases) was available from a previous study (Keller et al., 2007) and the remaining cases were processed in this study. The tumours were classified based on a revised Kiel classification scheme adapted to the dog (Fournel-Fleury et al., 1997; Ponce et al., 2004) by Dr. Paula Grest (Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich) with the support of Dr. Thierry Marchal (Ecole Nationale Vétérinaire de Lyon, F-69280 Marcy L'Etoile). Immunophenotypically, fifty-one cases were classified as B-cell lymphoma, thirty-one cases as T-cell lymphoma and eleven cases as Double-Negative (non-B- non-T-cell-) lymphoma.

### 4.7. Additional normal tissue blocks

Two tissue blocks were assembled with samples of selected canine tissues to provide whole sections of these organs free of histopathologic lesions. The tissues derived from the same animals sampled for the tissue arrays. The blocks were assembled and designated as follows:

1. ***Skin block:*** this block contained haired and glabrous skin from periocular and lip regions comprising the mucocutaneous transition zone from six different dogs.
2. ***Lymph node block:*** this block included four different lymph nodes from one healthy dog.

## 4.8. Immunohistochemistry (IHC)

### 4.8.1. Immunohistochemical protocols

All immunohistochemical applications were carried out in a wet chamber using the following standard immunoperoxidase protocol. Variable steps, such as antigen retrieval procedure as well as antibody dilution and incubation conditions, are indicated for each application separately in the following chapters.

Before sectioning, the blocks were either immersed in water and kept at -20 °C for 15 min (TMA blocks) or cooled to -20 °C (tissue blocks). Sections of 2.5 μm (whole sections) or 1.5 μm (TMAs) thickness were cut and transferred to positively charged glass slides (Erie Scientific Company, Thermo Scientific, Waltham, USA). The sections were dried over night at 37 °C and afterwards dewaxed and rehydrated in graded ethanol (100%, 95%, 70%) using a routine protocol. Antigen retrieval followed as described below. After heat-based antigen retrieval, the slides were always allowed to cool in the buffer solution for 10 min at RT before continuing with the immunohistochemical protocol. Incubation with primary antibodies against Bcl-2 family proteins followed as described below. To detect the primary antibody, the Detection Kit (Detection System, Peroxidase/AEC, Rabbit/Mouse, Dako REAL™, Dako Schweiz AG, Baar, Switzerland) was applied according to the manufacturer's instructions. Briefly, after incubation with the primary antibody, the sections were washed twice in PBS for 2 min. The secondary biotinylated antibody (Detection system, Dako REAL™) was applied for 10 min at RT. Washing steps were as described above. Endogenous peroxidase was inactivated by applying peroxidase blocking-solution (Detection system, Dako REAL™) to the sections for 10 min at RT. After washing steps, Streptavidin Peroxidase (HRP) (Detection system, Dako REAL™) was applied for 10 min at RT. Another washing step followed and the reaction was visualized by applying the chromogen 3-amino-9-ethyl-carbazole (AEC) (Detection system, Dako REAL™) for 10 min at RT, unless otherwise indicated. Finally, the slides were counterstained with hemalum and covered using KP-Tape (Klinipath, Duiven, The Netherlands). The amount of each added solution depended on the size either of the whole sections, or of the arrays and ranged from 200 to 400 μl per slide.

### 4.8.2. IHC of bacterial pellets

Twenty-one selected commercially available antibodies against human and/or murine Bcl-2 family proteins (Table 1) were tested for cross-reactivity and specificity by means of recombinant canine proteins using the standard protocol described above.

Following antigen retrieval methods (listed in increasing potency order) were carried out (Figure 3, no. 1 to 7):

1. No pre-treatment
2. Incubation with Proteinase K (final dilution 1:40; Dako REAL™) for 5 min at room temperature
3. Incubation with Proteinase K (final dilution 1:40; Dako REAL™) for 10 min at room temperature
4. Heat pre-treatment using a pressure cooker ("Pascal", Dako Cytomation, Dako Schweiz AG, Baar, Switzerland) and boiling the slides in a 1 in 10 dilution of acidic buffer (citrate buffer, pH 6.0, Dako REAL™) for 20 min at 98 °C
5. Heat pre-treatment using the "Pascal" pressure cooker and boiling the slides in a 1 in 10 dilution of alkaline buffer (Tris/EDTA buffer, pH 9.0, Dako REAL™) for 20 min at 98 °C
6. Heat and pressure pre-treatment using the "Pascal" pressure cooker and boiling the slides in a 1 in 10 dilution of acidic buffer (citrate buffer, pH 6.0, Dako REAL™) for 2 min at 125 °C
7. Heat and pressure pre-treatment using the "Pascal" pressure cooker and boiling the slides in a 1 in 10 dilution of alkaline buffer (Tris/EDTA buffer, pH 9.0, Dako REAL™) for 2 min at 125 °C

All primary antibodies were diluted 1 in 100 in Antibody Diluent (Dako REAL™) and they were incubated with two different conditions (1 h at RT and over night at 4 °C).

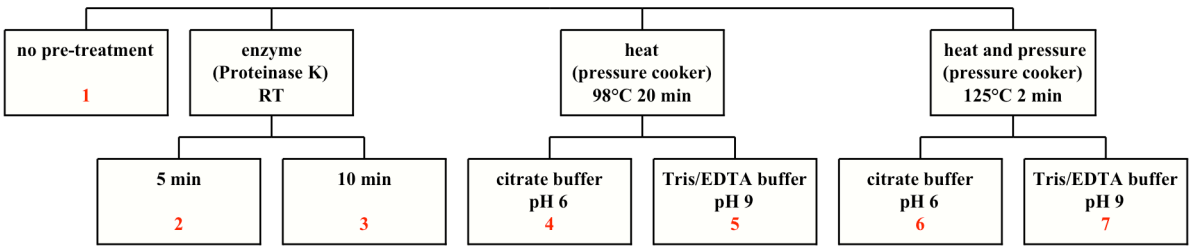



**Figure 3:** Schematic depiction of the seven different antigen retrievals methods (no. 1 to 7) used in this study

To evaluate immunohistochemical labelling of bacterial pellets, the intensity of the specific signal (i.e. labelling of the bacterial pellet expressing the corresponding GST-canine fusion protein) was categorized as absent, weak or strong. If all bacterial cores were labelled with similar intensity, this was considered as background labelling, which was scored as absent, weak, moderate or strong. Selected anti-Bcl-2 family member antibodies were deemed to cross-react with the corresponding canine homologue if the specific signal was more intense than background. Antibodies were considered as specific only if the bacterial pellet expressing the corresponding canine counterpart was labelled stronger than background. Non-specific labelling occurred if single additional bacterial cores were labelled.

***Antibody titre assessment***

The titre was determined for antibodies specifically cross-reacting with the corresponding canine protein in the system described above. Immunostaining of the bacterial pellets array was carried out using the antigen retrieval method and incubation condition showing the best ratio between specific signal and background. The primary antibodies were diluted 1 in 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, 10 000, 20 000 and 40 000 with exception of Mcl-1 antibody no. 4, which was purchased in a prediluted formula and for which dilution steps were adjusted. The highest antibody dilution retaining a clear-cut positive reaction (i.e. a clear-cut difference between specific signal and background labelling) was defined as the antibody titre. To make comparison between antibodies possible, the titre was expressed in ng/ul (Table 3).

### 4.8.3. IHC for Mcl-1 and Bcl-x in canine cells and tissues

The standard immunoperoxidase protocol described above was adapted for detection of these two proteins in canine tissues by varying the antigen retrieval procedure, as well as the dilution of the primary antibody and the AEC chromogen application time. Antigen retrieval procedures included pre-treatment omission, enzyme digestion for 10 min and heat pre-treatment at 98 °C using acidic or alkaline buffer (Figure 3 no. 1, 3, 4, 5). Antibody dilutions were varied between 1 in 50 and 1 in 200, time durations of AEC application ranged from 2 to 10 min. The standard incubation condition for primary antibodies was over night at 4 °C. The protocols were optimized using the cultured canine keratinocytes array and the skin and lymph node blocks. Final protocols were chosen based on the expected labelling patterns described in the literature. This aspect will be dealt with in detail in the discussion chapter.

The final protocol for immunohistochemical detection of Mcl-1 and Bcl-x in all canine tissues comprised a dilution of 1 in 100 of the primary antibody, as well as heat-based pre-treatment in alkaline buffer (Figure 3 no. 5) for Mcl-1 antibody no. 2 and pre-treatment omission (Figure 3 no. 1) for Bcl-x antibody no. 2. AEC was applied for 10 min or 3 min at RT to the sections incubated with Mcl-1 or Bcl-x antibodies, respectively. The bacterial pellets array was used as a positive control for each following immunohistochemical labelling experiment with canine tissues. As a negative control, the primary antibody was omitted, which resulted in a complete absence of signal in all tissues.

The following scoring system was used to score IHC labelling intensity in normal tissues and lymphoma samples: value 0=negative, 0-1=very faint, 1=faint, 1-2=very weak, 2=weak, 2-3=weak to moderate, 3=moderate, 3-4=moderate to strong, 4=strong, 4-5=strong to intense, 5=intense labelling.

To evaluate the distribution of B- and T-cell regions in normal canine lymph nodes, B- and T-cell immunophenotyping of sections of the lymph node block was performed. For this purpose, the monoclonal anti-human CD3 antibody (catalog no. M 725401, clone F7.2.38, Dako Schweiz AG, Baar, Switzerland), diluted 1 in 50, was used as a T-cell-marker. The monoclonal mouse anti-human CD79αcy antibody (catalog no. M705101, clone HM57, Dako Schweiz AG, Baar, Switzerland), diluted 1 in 250, was used as a B-cell-marker. For both antibodies, heat-based pre-treatment, consisting of boiling the slides in a 1 in 10 dilution of Tris/EDTA buffer for 20 min at 98 °C (Figure 3 no. 5) was used.

In the canine lymphoma arrays, each tumour was represented in four tissue cores. For evaluation of Mcl-1 and Bcl-x in the lymphoma samples, a mean value calculated as the arithmetical mean of scoring values determined for each individual tissue core available on

the arrays (i.e. not lost and containing representative tumour tissue) according to the scoring system, was determined for each tumour. Mean values and the standard deviation were calculated for the immunophenotypes and for all subtypes.

### 4.9. Preincubation of Mcl-1 antibody no. 2

To verify specificity of the IHC labelling, Mcl-1 antibody no. 2 preincubated with an excess of canine GST-Mcl-1 or canine GST-Bcl-x (as an additional control) was substituted for the primary antibody. This control was done for labelling of all canine tissues.

The recombinant GST-Mcl-1 and GST-Bcl-x fusion proteins expressed in bacteria were purified using Glutathione 4B Sepharose beads (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland). First, induced bacterial cultures (500 ml) were centrifuged at 6000g at 4 °C for 30 min and resuspended in 25 ml lysis buffer containing 50 mM Tris/HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5mM $MgCl_2$, 1mM DDT, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 0.3 µM Aprotinin, Leupeptin (5 µg/ml final concentration), Pepstatin-A (1 µg/ml final concentration). Afterwards, the bacteria were lysated by sonication twice for 2 min using Duty cycle 20% and Output maximum (Branson, Sonifier 250, G. Heinemann, Schwäbisch Gmünd, Germany). After centrifugation at 3500g at 4 °C for 30 min, the resulting supernatant was added to the sepharose beads prepared in advance. Preparation of sepharose beads was performed as follows: 1250 µl of beads were centrifuged at 500g at RT for 3 min, the supernatant was discarded and the beads were resuspended in 5 ml PBS. This procedure was repeated twice. After an additional centrifugation step, the supernatant was discarded and the beads were resuspended in 5 ml lysis buffer. After a final centrifugation at 500g at RT for 3 min, the supernatant was discarded and the supernatant of the sonicated bacterial lysates was added to the beads. The bacterial lysate-beads mixture was incubated for 1 h at 4 °C and mixed by inversion. Four washing steps with 5 ml lysis buffer and centrifugation at 500g at 4 °C for 3 min followed. For storing at -20 °C before further use, the GST-fusion protein-bound sepharose beads were resuspended in 2.1 ml lysis buffer and 2.9 ml 84% glycerol. Before starting the preincubation experiments, the purity of the proteins and their concentration were determined using SDS-PAGE gels stained with Coomassie-Blue (Roti®-Blue, Carl Roth GmbH 1 Co. KG, Karlsruhe, Germany) and the modified Bradford method (Uptima UPF8640, Interchim, Montlucon Cedey, France) according to the manufacturer's instructions. For preincubation, 3 ml of Mcl-1 antibody no. 2, diluted 1 in 100 in Antibody Diluent (Dako REAL™), were mixed to 500 µl GST-fusion protein-bound sepharose beads, containing either Mcl-1 or Bcl-x at a 100:1 antigen-antibody ratio. The mixtures were

incubated overnight at 4 °C and mixed by inversion. Centrifugation at 3000g at RT for 5 min followed. The preincubated antibody sample supernatant was stored at 4 °C and applied instead of the primary antibody in IHC runs, where appropriate.

IHC with the bacterial pellets array was used as a control for all preincubation experiments. A total removal of the signal from the core expressing Mcl-1 was considered as a working preincubation experiment. This was the case for each immunohistochemical labelling experiment performed.

For simplicity, the term "antibody preincubation" is used in this manuscript to indicate immunohistochemistry with Mcl-1 antibody no. 2 preincubated with GST-canine Mcl-1 protein.

### 4.10. Statistical analysis

For statistical analysis, mean scoring values for each tumour (calculated as the arithmetical mean of the values of all representative lymphoma tissue cores available for evaluation) were calculated for Mcl-1 and Bcl-x separately. In addition, a value "Mcl-1 corrected" was calculated for each tumour by subtracting the mean scoring value after preincubation from the mean scoring value without preincubation. Bravais-Pearson coefficients were calculated to determine if there was a linear correlation between the Mcl-1 and Mcl-1 corrected values versus the Bcl-x values. To determine if there were differences in the expression of Mcl-1 and Bcl-x between lymphoma immunophenotypes and/or subtypes, an analysis of variance (ANOVA) was carried out using the statistics software "R" (Development-Core-Team, 2008). In a first run, all immunophenotypes and subtypes were considered, in a second run (labelled "subtype2") the subtypes with low numbers of cases were pooled into one group. This second run comprised the subtypes centroblastic monomorphic, centroblastic polymorphic and marginal zone lymphoma (B-immunophenotype), pleomorphic and large granular lymphoma (T-immunophenotype), high-grade, medium sized lymphoma (Double-Negative) and all other subtypes pooled in one group. The limit for significance was set at a p value of 0.05.

# 5. Results

## 5.1. Establishing immunohistochemical protocols

### 5.1.1. Antibody testing

#### 5.1.1.1. Immunohistochemical testing of antibodies using formalin-fixed, bacterially-expressed canine Bcl-2 family proteins

A panel of selected commercially available anti-human and anti-mouse antibodies (Table 1) against the Bcl-2 family members Mcl-1, Bcl-x, Bcl-w, Bak, Bax, Noxa and Bad was tested immunohistochemically for anti-canine cross-reactivity and specificity using recombinant canine Bcl-2 family homologues. For this purpose, the bacterial pellets array (Figure 4), which contains formalin-fixed, paraffin-embedded bacteria expressing the corresponding GST-canine fusion proteins, as well as GST alone, was used.

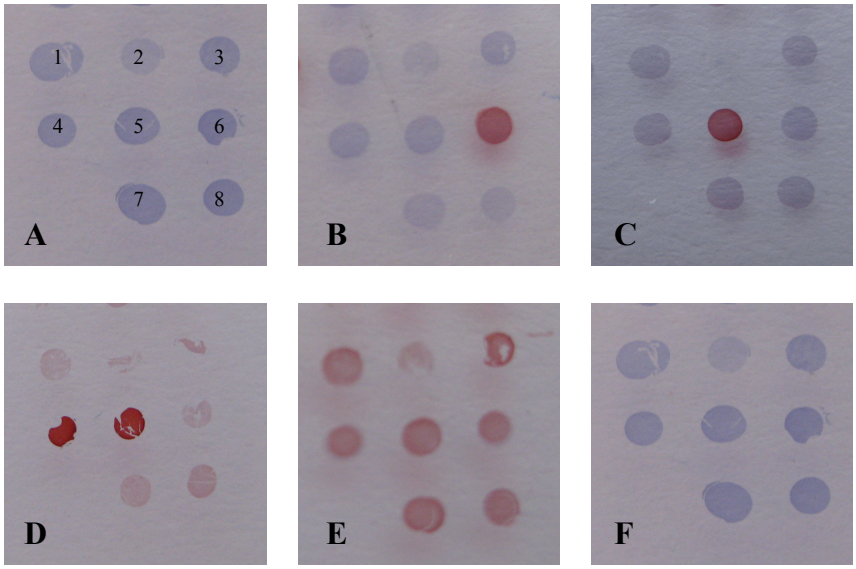
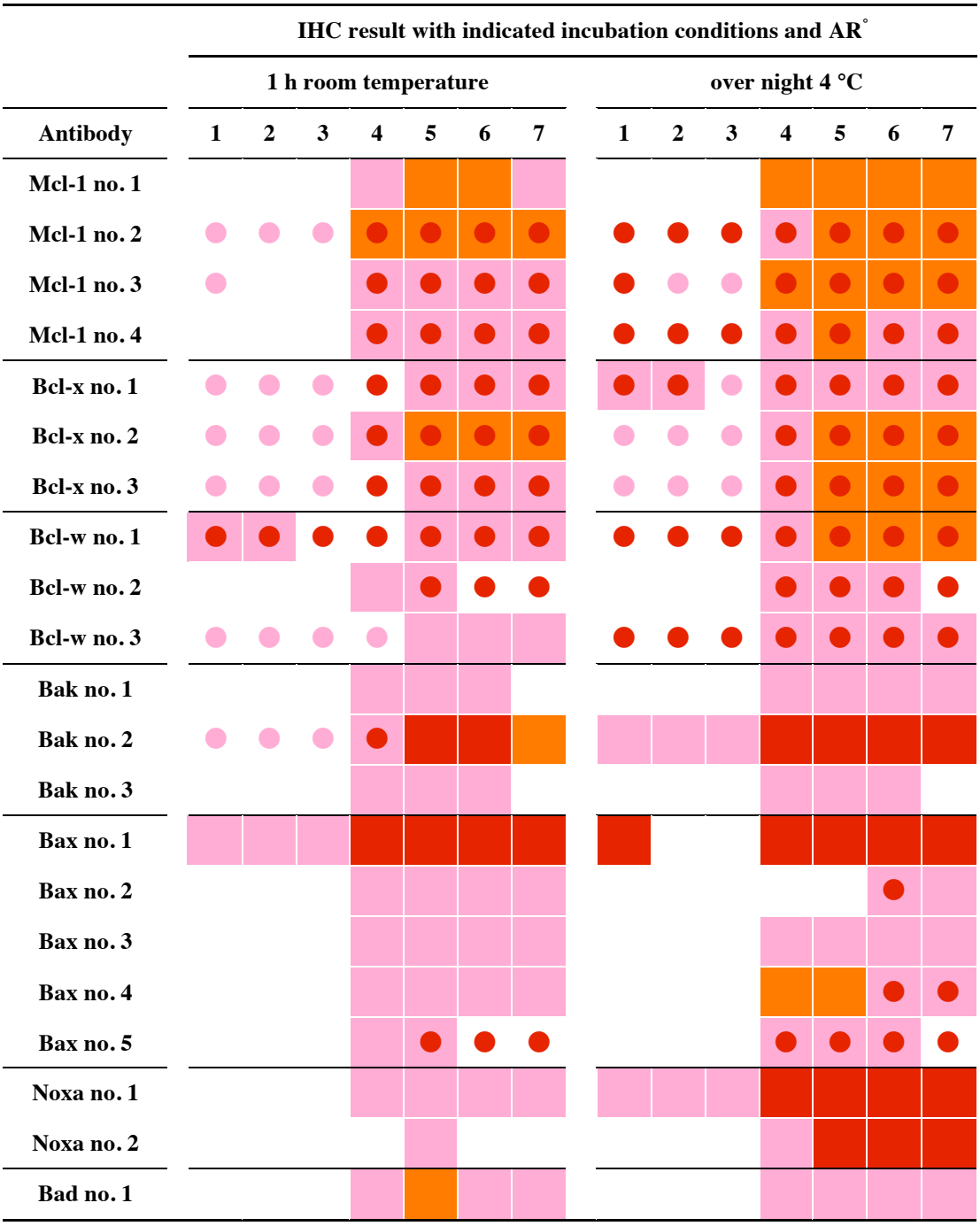



**Figure 4:** Immunohistochemical testing of antibodies against canine Bcl-2 family members using the bacterial pellets array
A. Array assembly. Bacterial cores are numbered according to the expressed protein: 1=GST-Bad, 2=GST-Bak, 3=GST-Bax, 4=GST-Bcl-w, 5=GST-Bcl-x, 6=GST-Mcl-1, 7=GST-Noxa, 8=GST; B. Specific labelling using Mcl-1 antibody no. 2; C. Specific labelling using Bcl-x antibody no. 2; D. Non-specific labelling using Bcl-w antibody no. 2; E. Background labelling using Noxa antibody no. 2; F. No labelling using Mcl-1 antibody no. 1.
A. Hemalum stain; B-F. Immunoperoxidase reaction, hemalum counterstain.




° AR=Antigen retrieval procedures 1 to 7 as described in Figure 3

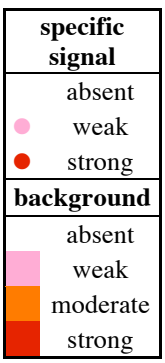



**Figure 5:** Immunohistochemical testing of anti-human and anti-mouse antibodies for cross-reactivity with the canine counterparts and effect of antigen retrieval and incubation conditions on specific signal and background labelling intensity.
Antibodies used: see Table 1.
Specific signal intensity was categorized as absent, weak or strong.
Background signal intensity was scored as absent, weak, moderate or strong.

Immunohistochemical antigen detection is often dependent on antigen demasking and incubation conditions optimized in dependence of specific antibody/antigen kinetics. Therefore, the antibody panel was tested using seven different antigen retrieval methods and two incubation conditions, as indicated in Figure 5. This figure shows the detailed results of this investigation. In summary, twelve of twenty-one anti-human and/or anti-mouse antibodies tested in this system were suitable for detection of the corresponding canine proteins. These comprised antibodies against Mcl-1, Bcl-x, Bcl-w, Bak and Bax (examples are shown in Figure 4B and 4C). However, none of the antibodies against the BH3-only proteins Noxa (n=2) and Bad (n=1) was found to cross-react with the canine counterpart in this system. Bcl-w antibody no. 2 cross-reacted with the corresponding bacterial pellet expressing GST-canine-Bcl-w, but also reacted non-specifically with GST-canine-Bcl-x (Figure 4D). For the remaining nine antibodies, cross-reactivity was not determined in this system and specificity was not assessable. They showed either no labelling at all or homogeneous background labelling of variable intensity.

The antigen retrieval and incubation conditions showed the following effects: only five of the twelve specific cross-reacting antibodies (Mcl-1 no. 2, Bcl-x nos. 1 to 3 and Bcl-w no. 1) yielded a specific signal in all fourteen antigen retrieval procedures and incubation conditions used (Figure 5). The remaining seven antibodies did not recognize their corresponding antigen in the majority of cases with non-heat-based pre-treatment. In general, a stronger background reaction was visible following heat-based antigen retrieval (AR procedures 4 to 7) than pre-treatment omission or enzyme digestion (AR procedures 1 to 3). Similarly, heat-based antigen retrieval was mostly associated with a stronger specific signal than non-heat-based antigen retrieval. The only exception was Bak antibody no. 2. In most instances, heat-based demasking increased non-specific background labelling with this antibody to such an extent that the specific signal was covered.

In several instances, over night incubation elicited stronger signals than incubation for 1 h. This effect was particularly prominent with antibodies against Bax (nos. 2 and 4).

Titres for all twelve specific, cross-reacting antibodies were determined, except for Bax antibody no. 2 (Lot exhausted) and are reported in Table 3. Most titres were in a range between 0.1 ng/µl and 1.2 ng/µl, with the exception of Bax antibody no. 5 and Bcl-w antibody no. 3, which had titres below 0.1 ng/µl and of Bak antibody no. 2, which had a titre of 4 ng/µl.

**Table 3:** Summary of results of antibody testing using IHC of formalin-fixed, paraffin-embedded bacterial pellets expressing selected canine recombinant Bcl-2 family members

| Antibody | Cross-reactivity | Specificity | Titre |
|---|---|---|---|
| **Mcl-1 no. 1** | no | - | - |
| **Mcl-1 no. 2** | yes | yes | 0.2 ng/µl |
| **Mcl-1 no. 3** | yes | yes | 1 ng/µl |
| **Mcl-1 no. 4** | yes | yes | 0.125 ng/µl |
| **Bcl-x no. 1** | yes | yes | 0.2 ng/µl |
| **Bcl-x no. 2** | yes | yes | 0.2 ng/µl |
| **Bcl-x no. 3** | yes | yes | 0.8 ng/µl |
| **Bcl-w no. 1** | yes | yes | 1.2 ng/µl |
| **Bcl-w no. 2** | yes | no° | not determined |
| **Bcl-w no. 3** | yes | yes | 0.024 ng/µl |
| **Bak no. 1** | no | - | - |
| **Bak no. 2** | yes | yes | 4 ng/µl |
| **Bak no. 3** | no | - | - |
| **Bax no. 1** | no | - | - |
| **Bax no. 2** | yes | yes | not determined |
| **Bax no. 3** | no | - | - |
| **Bax no. 4** | yes | yes | 0.4 ng/µl |
| **Bax no. 5** | yes | yes | 0.01 ng/µl |
| **Noxa no. 1** | no | - | - |
| **Noxa no. 2** | no | - | - |
| **Bad no. 1** | no | - | - |

- not assessable in the present experimental setting
° non-specific reaction with the bacterial pellet expressing Bcl-x

In conclusion, immunohistochemical testing with recombinant canine Bcl-2 family proteins allowed the identification of several antibodies against five family proteins (Mcl-1, Bcl-x, Bcl-w, Bak, Bax) for potential use in immunohistochemistry of tissues. For further immunohistochemical investigations in the present work, the focus was to detect Mcl-1 and Bcl-x protein in cells and tissues using Mcl-1 antibody no. 2 and Bcl-x antibody no. 2.

#### 5.1.1.2. Confirmatory Western blots

Selected IHC results were confirmed by Western blotting using bacterially-expressed GST-fusion proteins of the canine Bcl-2 family members Mcl-1, Bcl-x, Noxa and Bad, as well as GST alone, as antigens. Cross-reactivity with canine proteins was confirmed by this method for the two antibodies used in the second part of this study, i.e. polyclonal Mcl-1 antibody no. 2 and monoclonal Bcl-x antibody no. 2 (Figure 6A and 6B). The Mcl-1 antibody no. 2 and

the anti-GST-HRP antibody recognized a band of the expected molecular weight (64 kDa) corresponding to the GST-Mcl-1 fusion protein (Figure 6A). In contrast, immunoblotting of the same membrane with Mcl-1 antibody no. 1 did not reveal any band (not shown). Bcl-x antibody no. 2 labelled a band of 53 kDa with extracts from bacteria expressing GST-Bcl-x. Neither of specific antibodies Mcl-1 no. 2 and Bcl-x no. 2 showed any labelling with extracts from bacteria expressing GST alone (not shown).

A further analysis was done to ascertain that bacteria expected to express recombinant canine Noxa and Bad indeed expressed these molecules. A membrane with extracts from bacteria carrying the vector constructs for GST alone, GST-Noxa and GST-Bad was incubated with Anti-GST-HRP antibody (Figure 6C). A band of the expected size was labelled in each case (GST=27 kDa (Figure 6C lane 1), Bad=45 kDa (Figure 6C lane 2), Noxa=33 kDa (Figure 6C lane 3)). Western blots with both selected antibodies against Noxa (no. 1 and no. 2) however failed to label the corresponding bands (not shown). The selected antibody against Bad was not further tested.

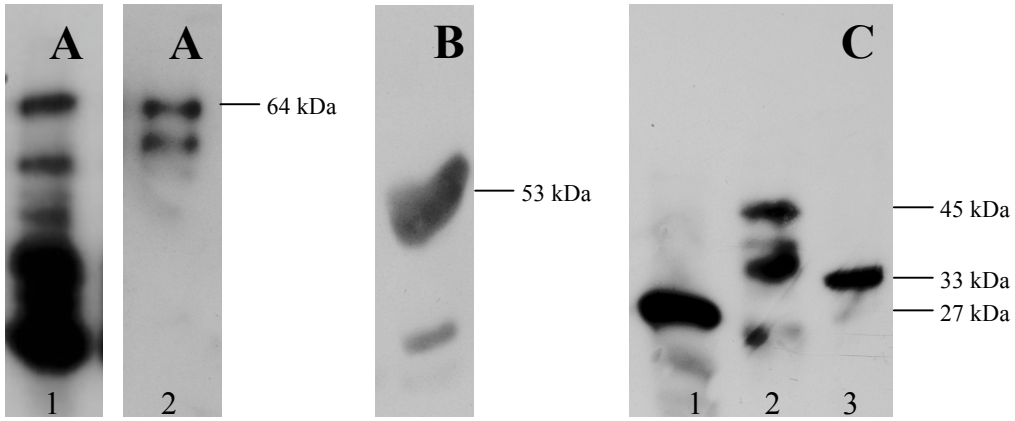



**Figure 6:** Confirmatory Western blots using bacterially-expressed canine Bcl-2 family proteins
In all cases bands of the expected size as indicated are labelled.
A. Protein extract from bacteria expressing GST-Mcl-1 labelled with the Anti-GST-HRP antibody (lane 1) and, after stripping of the membrane, with Mcl-1 antibody no. 2 (lane 2); B. Protein extract from bacteria expressing GST-Bcl-x labelled with Bcl-x antibody no. 2; C. Protein extracts from bacteria expressing GST alone (lane 1), GST-Bad (lane 2) and GST-Noxa (lane 3) labelled with the Anti-GST-HRP antibody.

In conclusion, Western blotting confirmed that Mcl-1 antibody no. 2 and Bcl-x antibody no. 2 recognize their canine target and that bacteria carrying the vector constructs for GST-Noxa and GST-Bad are expressing GST-fusion proteins of the expected size.

### 5.1.2. IHC for Mcl-1 and Bcl-x in cultured canine keratinocytes

IHC detection protocols for Mcl-1 and Bcl-x were established using non-irradiated and UV-irradiated cultured canine keratinocytes. This was the first step to establish immunohistochemical protocols suited to label these proteins in canine tissues with the selected antibodies.

***Mcl-1.*** Mcl-1 antibody no. 2 showed a diffuse cytoplasmic labelling with a distinct punctate pattern, which weakened following UV-irradiation, as expected (Figure 7A, B). Weakened Mcl-1 labelling was present at all points in time after UV-irradiation (i.e. 6, 12, 24 h), whereas the signal intensity at 0 h after UV-irradiation was similar to the signal intensity of the non-irradiated keratinocytes. Specificity of this labelling was confirmed with a preinucubation control. After antibody preincubation with GST-canine Mcl-1, the cytoplasmic signal intensity was clearly reduced, respectively disappeared widely (Figure 6C), while preincubation with GST-canine Bcl-x did not affect signal intensity at all (not shown). As a control for the preincubation step the IHC labelling of the bacterial pellets array with Mcl-1 preincubated antibody resulted in a complete abrogation of the specific signal.

***Bcl-x.*** Bcl-x antibody showed a similar expression pattern as the Mcl-1 antibody with distinct and consistent intracytoplasmic punctate labelling pattern, which did not change in intensity or distribution after irradiation, as expected (Figure 7D, E).

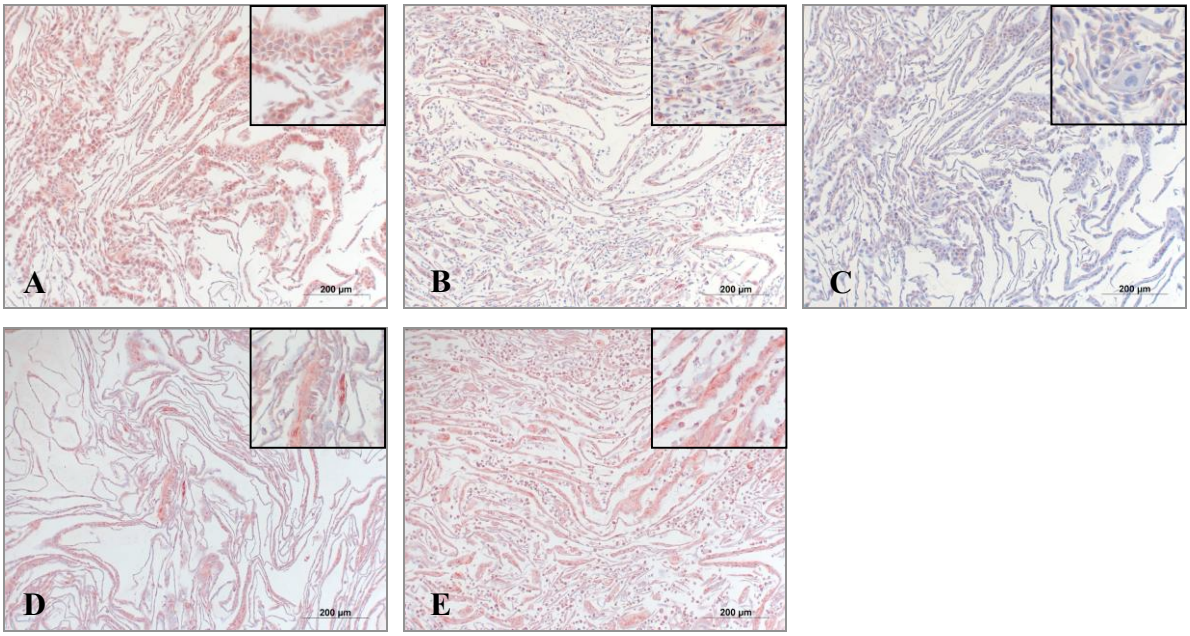



**Figure 7:** Mcl-1 and Bcl-x immunohistochemical labelling of cultured canine keratinocytes
A. IHC with Mcl-1 antibody no. 2 of non-irradiated cells; B. IHC with Mcl-1 antibody no. 2 of UV-irradiated cells after 6 h; C. IHC of non-irradiated cells after Mcl-1 antibody preincubation with GST-canine Mcl-1; D. IHC with Bcl-x antibody no. 2 of non-irradiated cells; E. IHC with Bcl-x antibody no. 2 of UV-irradiated cells after 6 h.
Inserts A-E: Double magnification.
A-E: Immunoperoxidase reaction, hemalum counterstain.

## 5.2. IHC for Mcl-1 and Bcl-x in canine tissues

To further optimize the detection protocols for Mcl-1 and Bcl-x in canine tissues, labelling with the two selected antibodies was done using whole sections from the canine skin block and lymph node blocks. The labelling patterns for these organs have consistently been described based on IHC in human tissues.

### 5.2.1. IHC for Mcl-1 and Bcl-x in whole sections of normal canine skin

***Mcl-1.*** When the Mcl-1 antibody was applied to sections of the skin block, it showed a consistently weak to moderate (intensity score: 2-3) labelling throughout all layers of the epidermis, excluding the unlabelled (0) stratum corneum (Figure 8A). These layers were best seen in the superficial epithelium of mucocutaneous regions (Figure 8), while they were difficult to differentiate in haired skin, which mostly displayed a thin epidermis. Epidermal keratinocytes were diffusely labelled over the whole cytoplasm. In the dermis, sweat glands and fibrocytes were labelled intensely (5), sebaceous glands and hair follicles were moderately to strongly (3-4), peripheral nerve fibers and endothelial cells moderately (3) and skeletal muscles were very weakly (1-2) immunostained for Mcl-1. When IHC was carried

out after antibody preincubation, the signal was strongly attenuated: it was completely negative in epithelial keratinocytes and fibrocytes, weak (2) in sweat and sebaceous glands and faint in endothelial cells and hair follicles (Figure 8B). In contrast, preincubation of the Mcl-1 antibody with an excess of GST-canine Bcl-x protein, performed as an additional control, did not reduce the signal intensity (Figure 8C).

***Bcl-x.*** In some specimens, the Bcl-x antibody elicited a faint immunostaining (1) of the basal layer keratinocytes, a weak to moderate labelling (2-3) of the spinous layer, a moderate reaction (3) of the granular layer, whereas the cornified layer was completely negative (Figure 8D). In other specimens, all epidermal layers were similarly labelled with the same intensity (2-3), except for the stratum corneum, which remained negative (not shown). Similarly, as with the Mcl-1 antibody, the epithelial cells showed a diffuse cytoplasmic labelling pattern. In the dermis, very weak (1-2) immunostaining for Bcl-x was detected in sweat glands, whereas sebaceous glands and endothelial cells were moderately labelled (3). Hair follicles showed a weak to moderate (2-3) signal and skeletal muscles displayed weak (2) immunoreactivity, whereas peripheral nerves were stained very faintly (0-1) and fibrocytes varied in the range between negative and weak (0/2).

**Table 4:** IHC for Mcl-1 and Bcl-x in whole sections of normal canine skin

| Skin region | Cell type | Labelling intensity° using indicated antibody | | |
|---|---|---|---|---|
| | | Mcl-1 no. 2 | Mcl-1 no. 2 preincubated* | Bcl-x no. 2 |
| Epidermis | Basal cell layer | 2-3 | 0/2† | 1/2-3 |
| | Spinous layer | 2-3 | 0/2 | 2-3 |
| | Granular layer | 2-3 | 0/2 | 3 |
| | Cornified layer | 0 | 0 | 0 |
| Dermis | Sweat glands | 5 | 2 | 1-2 |
| | Sebaceous glands | 3-4 | 2 | 3 |
| | Hair follicles | 3-4 | 0-1 | 2-3 |
| | Endothelium | 3 | 1 | 3 |
| | Muscles | 1-2 | 0 | 2 |
| | Fibrocytes | 5 | 0 | 0/2 |
| | Peripheral nerves | 3 | 0 | 0-1 |

° intensity scoring: 0=negative; 0-1=very faint; 1=faint; 1-2=very weak; 2=weak; 2-3=weak to moderate; 3=moderate; 3-4=moderate to strong; 4=strong; 4-5=strong to intense; 5=intense

* Mcl-1 antibody no. 2 preincubated with GST-canine Mcl-1

† 0/2=intensity varying between different tissue cores originating from different individuals within the indicated range [in this example comprising values from 0 to 2]

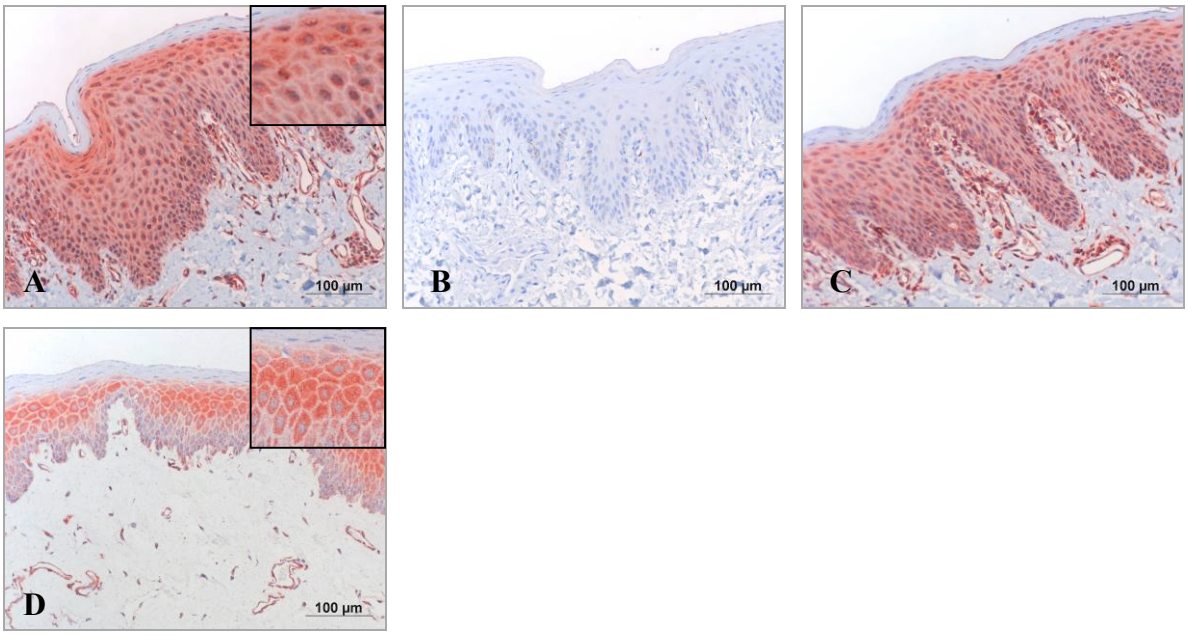



**Figure 8:** IHC for Mcl-1 and Bcl-x in whole sections of normal canine lip (mucocutaneous transition region)
A. IHC with Mcl-1 antibody no. 2; B. IHC after Mcl-1 antibody preincubation with GST-canine Mcl-1; C. IHC after Mcl-1 antibody preincubation with GST-canine Bcl-x; D. IHC with Bcl-x antibody no. 2.
Inserts A, D: Double magnification.
A-D: Immunoperoxidase reaction, hemalum counterstain.

### 5.2.2. IHC for Mcl-1 and Bcl-x in whole sections of normal canine lymph nodes

***Mcl-1.*** Lymphocytes of germinal centres were moderately to strongly (intensity score: 3-4) immunostained in sections of the lymph node block, whereas small lymphocytes comprising the mantle zone regions of lymph follicles showed no to weak (0/2) Mcl-1 immunoreactivity. Lymphocytes in interfollicular regions were weakly to moderately (2-3) immunostained throughout for Mcl-1. Mcl-1 immunoreactivity was diffuse over the entire cytoplasm. In some areas the signal appeared to be finely punctated. Remarkably, dendritic cells and macrophages in all regions of the lymph nodes were intensely Mcl-1 positive (5) and plasma cells were moderately (3) labelled. An example of a Mcl-1 labelled follicle is shown in Figure 9C (T- and B-cell immunotyping of a comparable region has been added as a guide to distinguish between compartments in Figures 9A and B, respectively). Antibody preincubation revealed a complete disappearance of the signal in lymphocytes, while in some of the dendritic cells and macrophages, a weak to moderate signal persisted (Figure 9D). Preincubation of Mcl-1 antibody no. 2 with GST-Bcl-x protein revealed the same labelling pattern as seen without preincubation (not shown).

***Bcl-x.*** The immunohistochemical expression pattern in lymph nodes was comparable to

Mcl-1 pertaining to the relative labelling intensity of the lymphocytes in the main lymph node compartments, but, in general, the labelling intensity was considerably weaker. Lymphoid follicle centres were stained very weakly (1-2), whereas labelling intensity varied between negative and very faint (0-1) in mantle zones and interfollicular regions (Figure 9E).

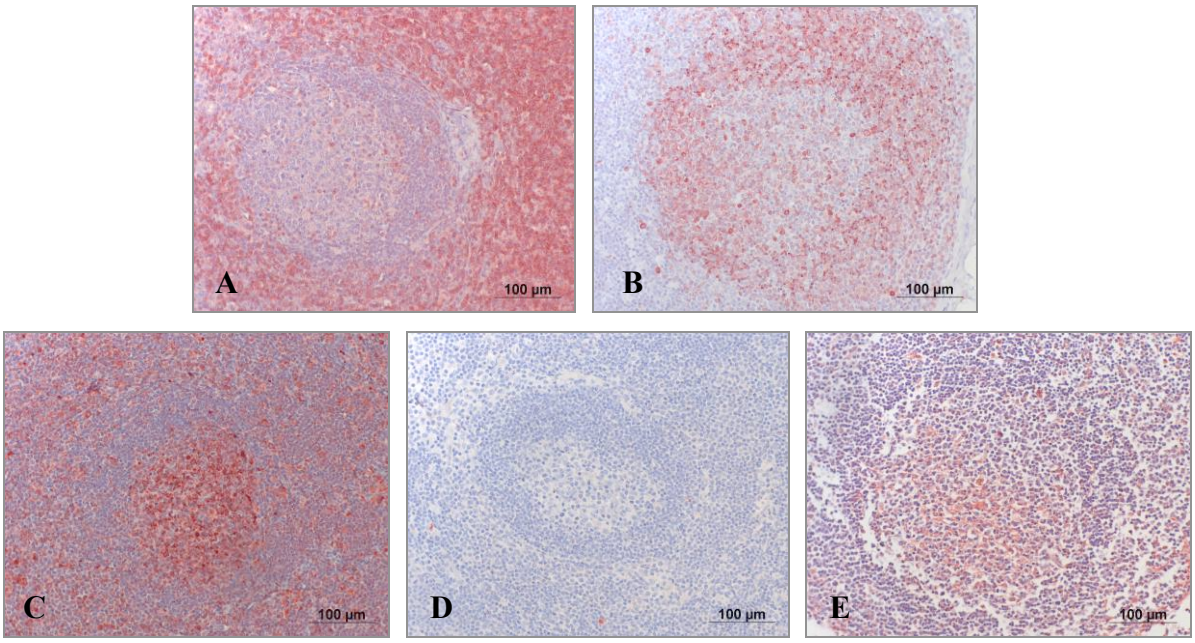



**Figure 9:** IHC for Mcl-1 and Bcl-x in whole sections of normal canine lymph nodes
An example of a secondary lymphoid follicle with germinal centre and surrounding mantle zone is shown.
A, B. Labelling with specific lymphocyte subtype markers: A. CD3 (T-cell marker); B. CD79a (B-cell marker); C. IHC with Mcl-1 antibody no. 2; D. IHC after Mcl-1 antibody preincubation with GST-canine Mcl-1; E. IHC with Bcl-x antibody no. 2.
A-E: Immunoperoxidase reaction, hemalum counterstain.

### 5.2.3. IHC for Mcl-1 and Bcl-x in microarrays of normal canine tissues

Optimized immunohistochemical protocols were used to carry out an immunohistochemical expression pattern analysis of Mcl-1 and Bcl-x in normal canine tissues comprised in tissue arrays. The tissues were selected for the study according to results of previous studies with human tissues.

The following general observations were made: Mcl-1 and Bcl-x labelling was restricted to the cytoplasm in all tissues examined. Mcl-1 labelling was relatively pronounced in epithelial cells of several tissues and in fibrocytes, dendritic cells and macrophages. Labelling of dendritic cells and macrophages did not completely disappear after antibody preincubation. Bcl-x labelling was frequently present in epithelial cells, but, in most instances, the signal intensity was weaker than Mcl-1 labelling. Variations of the labelling intensity between

different tissue cores, originating from different individuals, were observed for both proteins in several tissues. While after preincubation of the Mcl-1 antibody with GST-Bcl-x no signal reduction was observed, after antibody preincubation with GST-canine Mcl-1 a variable reduction of labelling intensity depending on the cell type was achieved.

The results of this investigation are presented in detail in Table 5 and are summarized in the following text. In general, it must be noted that not all findings described, were supported by all cores contained in the arrays because of occasional non-representative or lost tissue cores.

**Skin:**

***Mcl-1*** and ***Bcl-x.*** The labelling pattern seen in the tissue cores strongly resembled the expression pattern described above in whole sections of canine skin. After antibody preincubation, however, there were variations in the extent of signal intensity attenuation in different cores. Some cores showed complete signal disappearance in the epidermal layers, while in other cores, the epidermal labelling was only slightly attenuated.

**Digestive tract:**

***Mcl-1.*** In general, a relatively high expression intensity of Mcl-1 was seen in the digestive tract with exception of mucous acini in salivary glands, which were completely negative. Strong labelling of gastric glandular epithelium and villous enterocytes is shown in Figure 10A and B. Antibody preincubation was associated with a distinct attenuation of the IHC signal. In the exocrine pancreas cores, the signal was either absent or strong and in the latter case it persisted after preincubation.

***Bcl-x.*** The pattern and intensity of Bcl-x labelling was similar to Mcl-1, with the exception of Langerhans islet cells, which were more intensively labelled for Bcl-x than for Mcl-1 and of serous salivary gland acini and gastric chief cells, which were completely negative and exocrine pancreatic cells, which showed a faint labelling. Moderate to strong labelling of gastric parietal cells is shown in Figure 10I. Different cores of intestinal tissue showed variable labelling. Faint labelling of villous enterocytes is shown in Figure 10J.

**Respiratory tract:**

***Mcl-1.*** Tracheal and bronchial epithelium was weakly labelled with Mcl-1 antibody. Pneumocytes showed marked labelling intensity variations between cores ranging from negative to strong. This signal disappeared completely after antibody preincubation, while the signal in the tracheal epithelium was only slightly attenuated. Alveolar macrophages were strongly to intensely labelled.

***Bcl-x.*** A stronger labelling of the tracheal than the bronchial epithelium was observed.

Macrophages were also relatively strongly labelled.

**Cardiovascular system:**

***Mcl-1*** and ***Bcl-x.*** The myocardium was labelled with the same intensity for both proteins. Antibody preincubation resulted in a complete disappearance of the immunohistochemical signal.

**Reproductive organs:**

***Mcl-1.*** Weak to strong signal intensity was observed in the female reproductive organs. Figure 10C shows moderate to strong labelling of the endometrium and Figure 10D shows strong labelling of the mammary gland epithelium. All male reproductive tissues were relatively intensely stained for Mcl-1, particularly the prostate epithelium (Figure 10E), the spermatogonia and the Leydig cells in the testis (Figure 10F). In the different cell types of the testis a wide variation range between different cores was found. Antibody preincubation resulted in complete disappearance of the signal in the female genital organs, except for the epithelium of the mammary gland, where a faint signal persisted in some cores. In male genital organs a distinct attenuation of the signal was observed, except for the prostate epithelium, where a strong signal persisted in some cases.

***Bcl-x.*** Tissues of the reproductive tract were in most instances slightly weaker labelled for Bcl-x than for Mcl-1. Figure 10K shows weak to moderate labelling of the endometrium and Figure 10L shows a very weak labelling of the mammary gland epithelium. A remarkable finding was that, contrary to Mcl-1, no differences in labelling intensity between cores were observed in male genital organs.

**Musculoskeletal system:**

***Mcl-1.*** In skeletal muscles a faint immunohistochemical signal was detected, which was completely abrogated with preincubated antibody.

***Bcl-x.*** Variations between cores in the labelling intensity of skeletal muscles were observed in the range from faint to strong.

**Hematopoietic/lymphoid system:**

***Mcl-1.*** Lymphatic tissues were weakly to moderately labelled except for follicle centres of reactive secondary lymph follicles, which displayed a moderate to strong labelling intensity. Lymphocytes in the mantle zone of lymph nodes were labelled in the range from negative to weak. Antibody preincubation elicited complete abrogation of these signals. Thymocytes were virtually negative, the epithelioreticular cells of the thymus weakly to moderately immunostained and bone marrow cells very weakly labelled.

***Bcl-x.*** Lymphocyte labelling was distinctly weaker for Bcl-x than for Mcl-1, especially in

lymph nodes. In contrast, epithelioreticular cells of the thymus and bone marrow cells showed the same labelling intensity as for Mcl-1.

**Urinary tract:**

***Mcl-1*** and ***Bcl-x.*** The signal was mostly restricted to tubular epithelia (in distal tubuli stronger than proximal tubuli, as shown in Figure 10G and 10O) and to the urothelium. The latter showed moderate to strong labelling for Mcl-1 (Figure 10H) and intercore variations from weak to strong for Bcl-x (Figure 10P). The Mcl-1 signal was markedly reduced to completely abrogated after antibody preincubation.

**Endocrine organ:**

***Mcl-1.*** The thyroid follicular epithelium was intensely labelled. This labelling was almost completely abrogated after antibody preincubation.

***Bcl-x.*** A very weak signal of the thyroid follicular epithelium was detected.

**Central nervous system:**

***Mcl-1.*** In the central nervous system, neurons and ependymal cells were consistently strongly to intensely labelled. Strong to intense signal intensity was also observed in neurons and Purkinje cells of the brain, as well as in the ependyma of the spinal cord. Distinct intercore variations were detected in the neuroglia and neuropil. Antibody preincubation elicited a distinct signal attenuation, but in most cases complete abrogation failed to appear.

***Bcl-x.*** Bcl-x signal intensity was markedly reduced in comparison to Mcl-1 in neurons. In contrast, the neuropil showed a stronger signal, whereas intercore variations in the labelling intensity of neuroglia were also observed with Bcl-x.

**Table 5:** IHC for Mcl-1 and Bcl-x in microarrays of normal canine tissues

| Organ/tissue | Cell type | Labelling intensity° using indicated antibody | | |
|---|---|---|---|---|
| | | Mcl-1 no. 2 | Mcl-1 no. 2 preincubated* | Bcl-x no. 2 |
| **Skin** | | | | |
| Epidermis | Basal cell layer | 2-3 | 0/2† | 1/2-3 |
| | Spinous layer | 2-3 | 0/2 | 2-3 |
| | Granular layer | 2-3 | 0/2 | 3 |
| | Cornified layer | 0 | 0 | 0 |
| **Digestive** | | | | |
| Salivary glands | Serous acini | 5 | 1-2 | 0 |
| | Mucous acini | 0 | 0 | 0 |
| | Excretory duct | 3/5 | 0/1 | 2 |
| Stomach | Parietal cells | 4 | 0/1 | 3-4 |
| | Chief cells | 4 | 0 | 0 |
| Intestine | Enterocytes | 4 | 0 | 1/4 |
| Liver | Hepatocytes | 2/4 | 0/1 | 2/4 |
| Pancreas | Exocrine cells | 0/5 | 0/4 | 1 |
| | Islets of Langerhans | 1-2 | 0 | 2-3 |
| **Respiratory** | | | | |
| Trachea | Epithelium | 2 | 1-2 | 2-3 |
| | Chondrocytes | 0 | 0 | 0 |
| Bronchi | Epithelium | 1-2 | n.a.Δ | 1 |
| Alveoli | Pneumocytes | 0/4 | 0 | 0 |
| | Alveolar macrophages | 4-5 | 0/2 | 2/4 |
| **Cardiovascular** | | | | |
| Heart | Myocardium | 1-2 | 0 | 1-2 |
| **Reproductive** | | | | |
| Ovary | Germinal epithelium | 2 | 0 | 3 |
| | Corpus luteum | 4 | n.a. | 3 |
| Uterus | Endometrium | 3-4 | 0 | 2-3 |
| Vagina | Epithelium | 2-3 | 0 | 0/2 |
| Mammary gland | Epithelium | 4 | 0/1 | 1-2 |
| Prostate | Epithelium | 5 | 0/4 | 3-4 |
| Testis | Spermatogonia | 0/5 | 0/1 | 1-2 |
| | Spermatocytes | 0/3 | 0/2 | 1-2 |
| | Spermatids | 0/3 | 0/2 | 1-2 |
| | Sertoli cells | 1/3 | 0/1 | 1-2 |
| | Leydig cells | 5 | 0/2 | 3-4 |
| **Musculoskeletal** | | | | |
| Skeletal muscle | Myotubules | 1 | 0 | 1/4 |
| **Hematopoietic/ lymphoid** | | | | |
| Spleen | White pulp lymphocytes | 2 | 0 | 0 |
| | Red pulp cells | n.a. | 0 | 1-2 |
| Tonsil/lymph node | Germinal centre lymphocytes | 3-4 | 0 | 1-2 |
| | Interfollicular lymphocytes | 2-3 | 0 | 0-1 |
| | Mantle zone lymphocytes | 0/2 | 0 | 0-1 |
| Thymus | Cortical thymocytes | 0-1 | 0 | 0 |

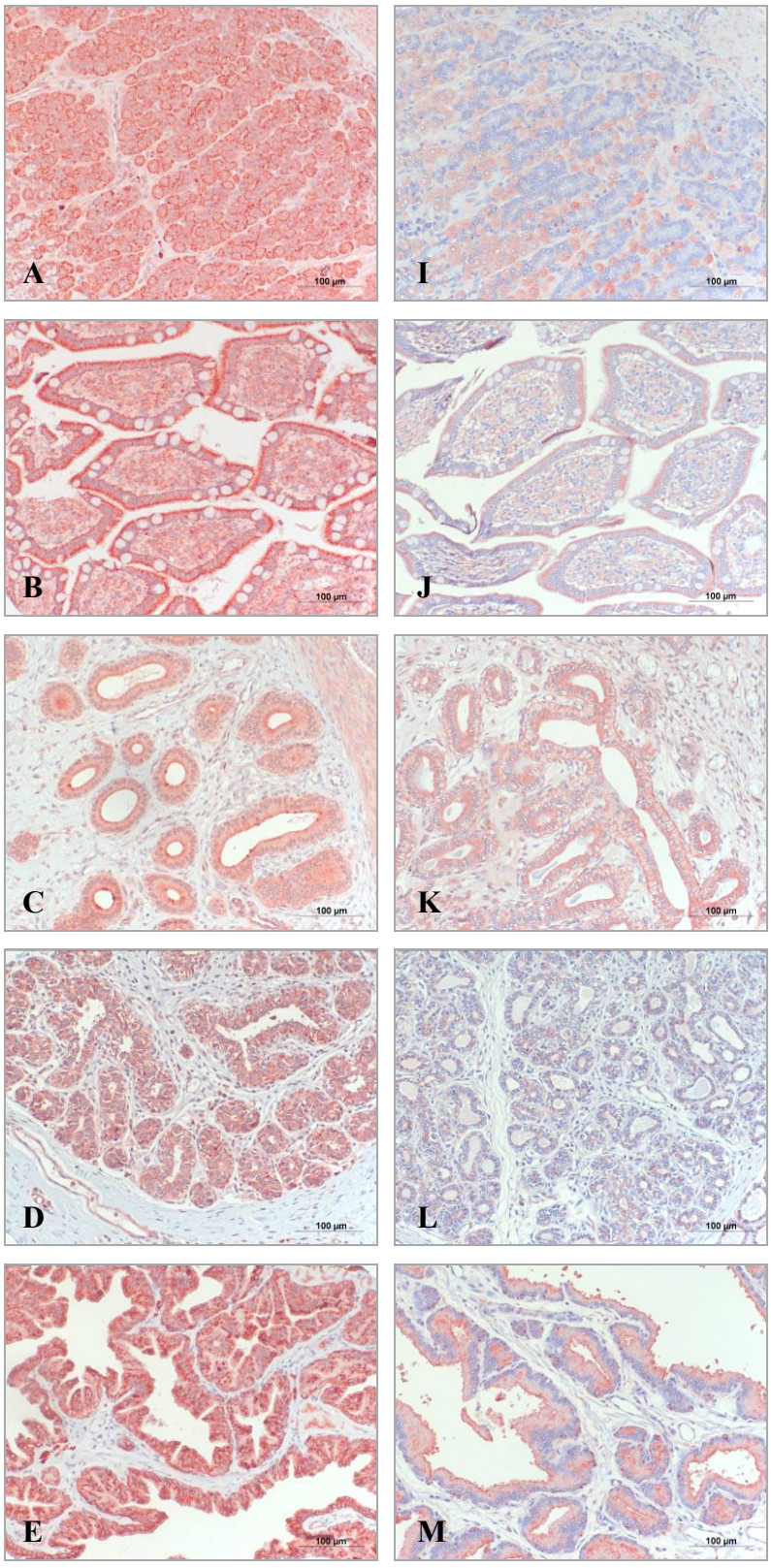
| | | | | |
|---|---|---|---|---|
| | Medullary thymocytes | 0-1 | 0 | 0 |
| | Epithelioreticular cells | 2-3 | 0 | 2-3 |
| Bone Marrow | Myeloid precursors | 1-2 | 0 | 1-2 |
| | Erythrocyte precursors | 1-2 | 0/1 | 1-2 |
| | Megakaryocytes | 1 | 0 | 1 |
| **Urinary tract** | | | | |
| Kidney | Glomeruli | 1 | 0 | 0 |
| | Proximal tubuli | 3-4 | 0/1 | 2-3 |
| | Distal tubuli | 4-5 | 0/1 | 3-4 |
| Bladder | Epithelium | 3-4 | 1 | 2/4 |
| **Endocrine** | | | | |
| Thyroid | Follicular epithelium | 5 | 0/1 | 1-2 |
| **Central nervous system** | | | | |
| Brain | | | | |
| Cortex | Large neurons | 4-5 | 2-3 | 1-2 |
| | Neuropil | 0/1 | 0 | 2-3 |
| | Neuroglia | 0/5 | 0 | 0/5 |
| | Axons in white matter | 2/4 | 1-2 | 1/3 |
| Cerebellum | Purkinje cells | 4-5 | 1-2 | 0/1 |
| | Neurons | 4-5 | 1-2 | 1-2 |
| Spinal cord | Neurons | 4-5 | 2-3 | 1-2 |
| | Neuroglia | 0/5 | 0 | 0/5 |
| | Myelin fibers | 2/4 | 1-2 | 1/3 |
| | Neuropil | 0/1 | 0 | 2-3 |
| | Ependyma | 4-5 | 1-2 | 3-4 |

° intensity scoring: 0=negative; 0-1=very faint; 1=faint; 1-2=very weak; 2=weak; 2-3=weak to moderate; 3=moderate; 3-4=moderate to strong; 4=strong; 4-5=strong to intense; 5=intense

* Mcl-1 antibody no. 2 preincubated with GST-canine Mcl-1

† 0/2=intensity varying between different cores originating from different individuals within the indicated range [in this example comprising values from 0 to 2]

Δ n.a.=(tissue) not available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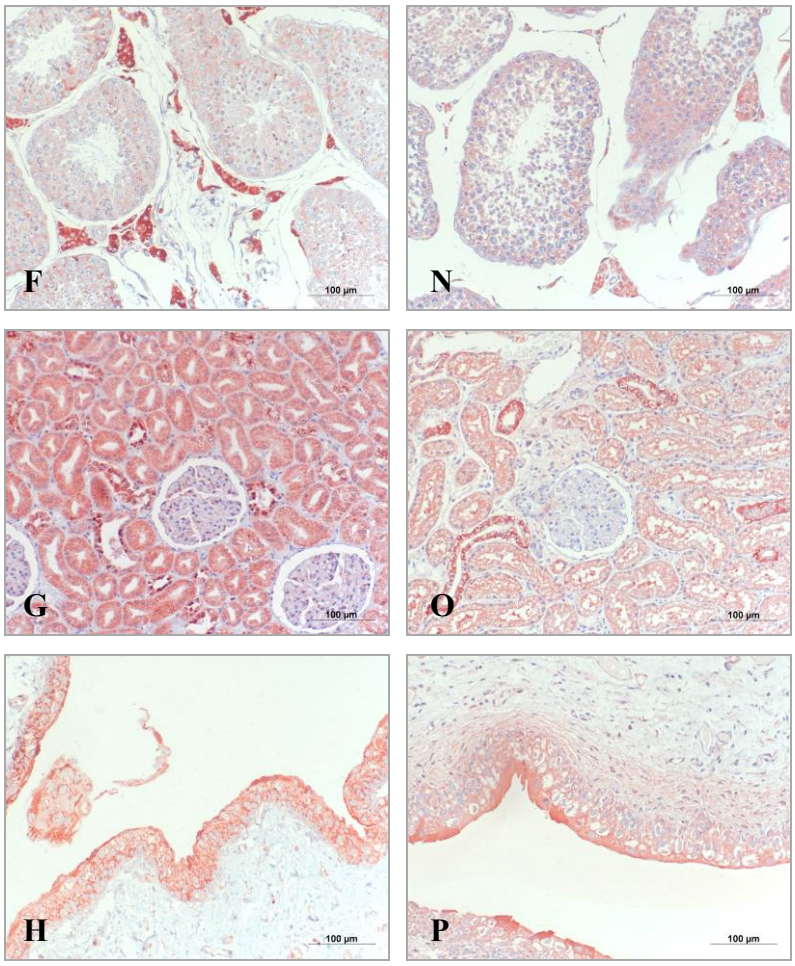


**Figure 10:** IHC for Mcl-1 and Bcl-x in microarrays of normal canine tissues
A-H. IHC with Mcl-1 antibody no. 2; I-P. IHC with Bcl-x antibody no. 2
A, I. Gastric mucosa; B, J. Small intestinal mucosa (villi); C, K. Endometrium; D, L. Mammary gland; E, M. Prostatic glands; F, N. Tubuli contorti of the testis; G, O. Renal cortex; H, P. Mucosa of the urinary bladder.
A-P: Immunoperoxidase reaction, hemalum counterstain.

### 5.2.4. IHC for Mcl-1 and Bcl-x in microarrays of canine lymphoma samples

Optimized immunohistochemical protocols were applied to investigate the expression of Mcl-1 and Bcl-x in a set of ninety-three archival canine malignant lymphoma samples assembled into tissue arrays. The tumours were classified according to a revised Kiel-classification. The distribution frequency of the various lymphoma subtypes in this collective can be conveyed from Table 6, where Mcl-1 and Bcl-x expression score averages for the immunophenotypes and for the major subtypes are reported. The mean scoring values for each tumour (calculated as the arithmetical mean of the scoring values determined for each individual tissue core available on the arrays) are reported in Appendix A. In general, the majority of tumour cells in a given core were labelled with a relatively homogeneous

intensity. The scoring values derived from the individual tissue cores for a single tumour mostly were identical or did not strongly differ from each other. Bcl-x labelling was distinctly weaker than Mcl-1 labelling. For both proteins, labelling was restricted to the cytoplasm of the tumour cells and its distribution was diffuse and finely stippled to coarsely granulated. Representative labelling for a number of tumours is shown in Figure 11. In a very few cases there was additionally a diffuse labelling of the nucleus. This labelling mostly occurred in cores containing not well-preserved tissue and it was not included in the scoring. Preincubation of Mcl-1 antibody with GST-Mcl-1 elicited complete disappearance of the signal in neoplastic lymphocytes in most instances. In a small number of cases a slight labelling persisted. This residual labelling was scored as very faint (intensity scoring: 0-1) in seventeen tumours and as faint (1) in five further tumours. Residual labelling was stronger than faint (1) in two instances: in a B-cell centroblastic polymorphic lymphoma (Appendix A, tumour no. 23) its mean scoring value was 1.2 (the intensity scoring without preincubation was 4) and in a Double-Negative, high-grade, medium-sized lymphoma (Appendix A, tumour no. 84) it was 3.9 (while the intensity scoring without preincubation was 4.5). To tentatively correct for this residual labelling observed after antibody preincubation, a value "Mcl-1 corrected" was calculated for each tumour by subtracting the mean scoring value after preincubation from the mean scoring value without preincubation. These corrected values are reported in both relevant tables (Table 6 and Appendix A). Contrary to GST-Mcl-1, GST-Bcl-x preincubation of the Mcl-1 antibody no. 2 did not affect the immunohistochemical signal in any way.

In addition to the neoplastic cells, dendritic cells and macrophages, tentatively identified based solely on morphological criteria and present in variable numbers in the tumours, showed a strong labelling for Mcl-1 similarly as described above in normal tissues (an example can be seen in Figure 11B). This signal was variably abrogated after antibody preincubation with GST-Mcl-1, while it was unaffected by preincubation with GST-Bcl-x. In some cases it disappeared completely, in other cases its intensity did not change noticeably. This strong labelling of dendritic cells and macrophages was disregarded while scoring the lymphoma tissues.

**Table 6:** IHC for Mcl-1 and Bcl-x in microarrays of canine lymphomas. Average and standard deviation of scoring values for the indicated immunophenotypes and subtypes

| Lymphoma subtype | Average and standard deviation of immunohisto-chemical expression scores for indicated protein | | |
|---|---|---|---|
| | Mcl-1 | Mcl-1 corrected[°] | Bcl-x |
| **B-Immunophenotype (n[†]=51)** | **3.5 (1.0)** | **3.3 (1.1)** | **2.5 (1.0)** |
| centroblastic monomorphic (n=10) | 3.9 (0.8) | 3.7 (0.9) | 2.1 (0.7) |
| centroblastic polymorphic (n=16) | 3.2 (1.2) | 3.0 (1.2) | 2.4 (1.1) |
| marginal zone (n=11) | 3.0 (0.6) | 2.8 (0.9) | 2.2 (0.8) |
| lymphoplasmacytic (n=2) | 4.1 (1.3) | 4.2 (1.1) | 1.7 (1.5) |
| Burkitt (n=2) | 3.7 (1.9) | 3.4 (2.2) | 2.8[*††] |
| other subtypes[Δ] (n=5) | 4.5[*] (0.6) | 4.4[*] (0.5) | 3.9 (0.7) |
| not classifiable (n=5) | 3.9[*] (1.2) | 3.6 (0.9) | 2.7 (1.0) |
| **T-Immunophenotype (n=31)** | **2.8 (1.1)** | **2.6 (1.1)** | **2.3 (1.0)** |
| pleomorphic (various sizes) (n=15) | 2.5 (1.0) | 2.4 (1.0) | 2.3 (0.9) |
| large granular lymphoma (n=5) | 4.2 (1.3) | 4.0[*] (1.2) | 2.1 (1.0) |
| lymphoblastic (n=2) | 2.0 (0.1) | 2.0 (0) | 3.5[*††] |
| other subtypes[°°] (n=4) | 2.9 (0.9) | 2.6 (1.1) | 2.6 (1.5) |
| not classifiable (n=5) | 2.5 (0.5) | 2.5 (0.5) | 2.0 (1.3) |
| **Double Negative-Immunophenotype (n=11)** | **2.3 (1.2)** | **1.9 (1.6)** | **3.1 (1.2)** |
| high-grade, medium sized (n=5) | 3.8 (1.0) | 2.9 (1.5) | 2.8 (1.6) |
| other subtypes[**] (n=3) | 2.1 (0.9) | 1.7 (1.3) | 3.4 (0.3) |
| not classifiable (n=3) | 4.0[*] (1.4) | 4.0[*] (1.4) | 3.4 (1.5) |

[°] Mcl-1 corrected=score value after IHC with antibody Mcl-1 no. 2 minus score value after IHC with preincubated Mcl-1 antibody

[*] in one case cores not available

[†] n=number of cases

[Δ] including one immunoblastic B-cell lymphoma, one plasmablastic B-cell lymphoma, one diffuse large T-cell lymphoma, one follicular T-cell lymphoma and one small T-cell lymphoma.

[°°] including one plasmacytoid T-cell lymphoma, one cytotoxic T-cell lymphoma, one small clear cell type T-cell lymphoma and one case of mycosis fungoides

[**] including one plasmacytoid Double-Negative lymphoma, one immunoblastic Double-Negative lymphoma and one case of mycosis fungoides

[††] standard deviation not available (only one case available)

The results of the statistical analysis of the IHC were as follows: the expression data for Mcl-1 and Bcl-x were normally distributed and the variance was homogeneous across the whole range of values. The Bravais-Pearson correlation coefficients were 0.09 for the comparison Mcl-1/Bcl-x and 0.02 for the comparison Mcl-1 corrected/Bcl-x, indicating that there is no linear correlation between these pairs of data sets. Results of the analysis of variance considering immunophenotypes and lymphoma subtypes are reported in Table 7. This analysis revealed that the immunophenotype has an influence on the expression of Mcl-1 ($p=0.006$) and Mcl-1 corrected ($p=0.02$), but not on the expression of Bcl-x ($p=0.12$). Pairwise comparisons between the immunophenotypes showed that the expression of Mcl-1 and Mcl-1 corrected significantly differed in B-cell and T-cell lymphomas ($p=0.004$ and

p=0.02, respectively). Pairwise comparisons between the subtypes showed that the expression of Mcl-1 significantly differed between T-cell large granular lymphomas and B-cell marginal zone, B-cell centroblastic polymorphic and T-cell pleomorphic lymphomas (p=0.01, p=0.03, p=0.04).

**Table 7:** Analysis of variance (ANOVA) for immunohistochemical expression scoring values of Mcl-1 and Bcl-x in canine lymphomas

| | **Degree of freedom** | **Sum of Squares** | **Mean of Squares** | **F value** | **P value** |
|---|---|---|---|---|---|
| Computation including all subtypes | | | | | |
| **Mcl-1 expression** | | | | | |
| **immunophenotype** | 2 | 10.874 | 5.437 | 5.5873 | 0.005732** |
| **subtype** | 17 | 27.461 | 1.615 | 1.6601 | 0.073939 |
| **immunophenotype:subtype** | 3 | 5.373 | 1.791 | 1.8407 | 0.148347 |
| **residuals** | 66 | 64.222 | 0.973 | | |
| **Mcl-1 expression corrected** | | | | | |
| **immunophenotype** | 2 | 9.857 | 4.929 | 4.1566 | 0.01994 |
| **subtype** | 18 | 29.374 | 1.632 | 1.3763 | 0.17356 |
| **immunophenotype:subtype** | 2 | 7.514 | 3.757 | 3.1684 | 0.04854* |
| **residuals** | 66 | 78.258 | 1.186 | | |
| **Bcl-x expression** | | | | | |
| **immunophenotype** | 2 | 4.751 | 2.376 | 2.2036 | 0.1188 |
| **subtype** | 18 | 22.794 | 1.266 | 1.1747 | 0.3088 |
| **immunophenotype:subtype** | 3 | 1.949 | 0.65 | 0.6027 | 0.6156 |
| **residuals** | 63 | 67.916 | 1.078 | | |
| Computation with pooled subtypes | | | | | |
| **Mcl-1 expression** | | | | | |
| **immunophenotype** | 2 | 10.874 | 5.437 | 5.7805 | 0.004520** |
| **subtype2** | 6 | 21.814 | 3.636 | 3.8654 | 0.001944 |
| **residuals** | 80 | 75.243 | 0.941 | | |
| **Mcl-1 expression corrected** | | | | | |
| **immunophenotype** | 2 | 9.857 | 4.929 | 4.1384 | 0.01994* |
| **subtype2** | 6 | 19.869 | 3.312 | 2.7806 | 0.01656* |
| **residuals** | 80 | 95.277 | 1.191 | | |
| **Bcl-x expression** | | | | | |
| **immunophenotype** | 2 | 4.751 | 2.376 | 2.1502 | 0.1233 |
| **subtype2** | 6 | 6.481 | 1.08 | 0.9776 | 0.4461 |
| **residuals** | 78 | 86.179 | 1.105 | | |

*/**=significant/highly significant interaction

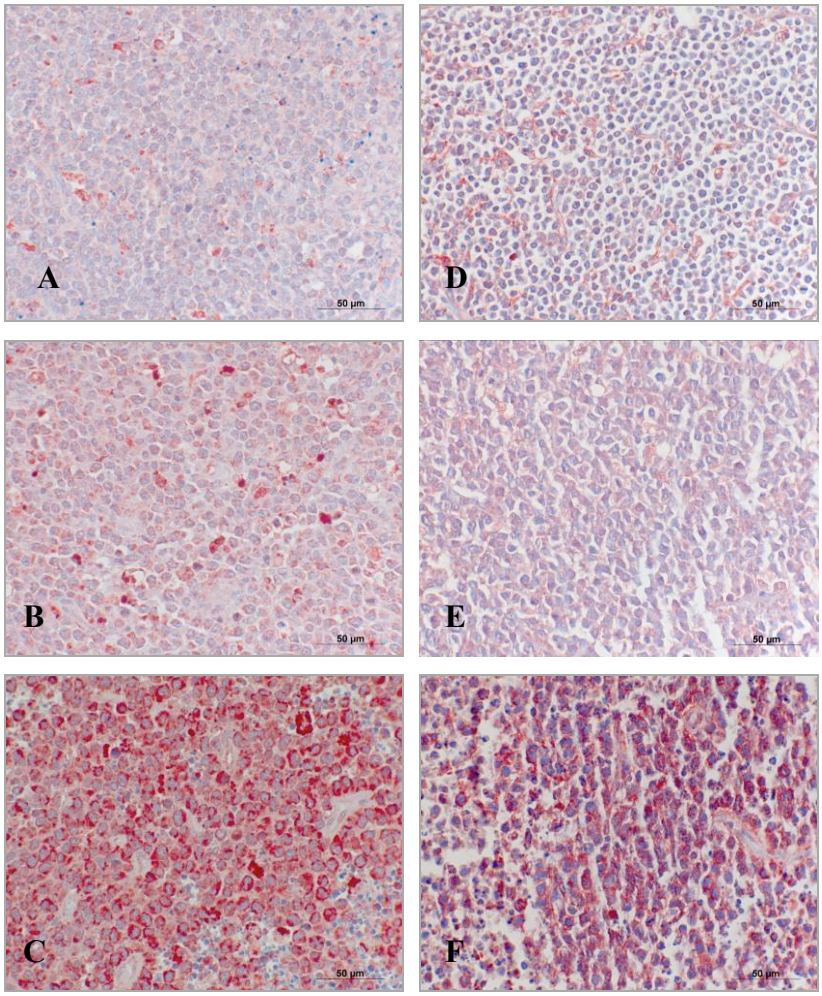


**Figure 11:** IHC for Mcl-1 and Bcl-x in microarrays of canine lymphoma samples
A-C. IHC with Mcl-1 antibody no. 2; D-F. IHC with Bcl-x antibody no. 2.
A, D. Pleomorphic T-cell lymphoma, indicated as tumour no. 56 in Appendix A; B, E. Centroblastic monomorphic B-cell lymphoma, indicated as tumour no. 6 in Appendix A; C, F. Diffuse large B-cell lymphoma, indicated as tumour no. 44 in Appendix A.
A. Intensity score: 1; B. Intensity score: 3; C. Intensity score: 5; D. Intensity score: 1; E. Intensity score: 2; F. Intensity score: 5.
A-F. Immunoperoxidase reaction, hemalum counterstain.

# 6. Discussion

In this study, a system based on immunohistochemistry of formalin-fixed, paraffin-embedded bacteria expressing recombinant canine Bcl-2 family proteins was used to test commercially available antibodies against heterologous Bcl-2 family antigens. Anti human and/or anti mouse antibodies were analysed for their suitability for immunohistochemistry of canine tissues. In accordance with findings from a previous investigation, in which this system was described for the first time, it proved suitable for this purpose (Keller et al., 2007). In the previous study, only antibodies against p53 were tested, whereas in the present work, the system has been extended to include the Bcl-2 family proteins Mcl-1, Bcl-x, Bcl-w, Bak, Bax, Noxa and Bad. In addition, the effect of diverse antigen retrieval methods was examined.

Of a panel of twenty-one antibodies against these selected Bcl-2 family members, twelve antibodies were found to specifically cross-react with the corresponding canine homologue. In the present bacterial pellets array, based on specific reactions with at least one of the antibodies tested, proper expression of the expected antigen was demonstrated for five molecules, i.e. Mcl-1, Bcl-x, Bcl-w, Bak and Bax. In addition, Western blot analysis confirmed this finding for two proteins (Mcl-1 and Bcl-x), whose expression in canine tissues was studied in the second part of this work. None of the antibodies against the two additional molecules, Noxa and Bad, showed any specific labelling of the bacteria contained in the array. Despite this fact, both proteins were likely expressed, as indicated by the detection of bands of the expected size in GST-immunoblotting of the corresponding bacterial extracts. Both antibodies in the panel, however, failed to label the band putatively representing Noxa in Western blots, indicating that they may not cross-react with canine Noxa. Immunoblotting with the specific Bad antibody was omitted in the present work. Thus, in a future study further antibodies against Noxa and Bad should be added and further confirmatory Western blots might be necessary to determine unequivocally that these proteins are expressed in the array and to select specific, cross-reacting antibodies.

Monoclonal antibodies not cross-reacting due to epitope sequence discrepancies between human or mouse and dog are likely to be easily identified with this system. This may be the case for Mcl-1 antibody no. 1, whose lack of cross-reaction towards the canine protein was confirmed by Western blot analysis. In addition, the system allows detection of antibodies reacting non-specifically, as it is the case for Bcl-w antibody no. 2, which labelled the correct corresponding homologue and additionally Bcl-x. Ideally, an array containing bacteria

expressing all known members of a protein family would warrant that non-specific reactions are excluded with a certain degree of reliability.

The test system used in this study to assess cross-reactivity and specificity of antibodies has potential advantages because it is similar to the application for which the antibodies are selected, i.e. the detection of antigens in fixed tissues. In contrast, in Western blot analysis the antigens are linearized, which may not always be the case with fixed antigens. Antigen retrieval is another important aspect where similarity between the test system and final applications may apply. This term includes a number of different procedures widely used in immunohistochemistry to demask antigens in tissues not accessible to the antibodies due to fixation-dependent protein cross-linking (Yamashita, 2007). Antigen retrieval experiments using formalin-fixed peptides either alone or peptides combined with a protein-mix showed that the latter more accurately mimic the behaviour of certain antigens in tissues, likely because of the increased occurrence of fixative-dependent cross-linking (Sompuram et al., 2004). In this respect, the system used in the present study seems to be ideal, since the specific proteins expressed are mixed with additional proteins present in the bacterial cytoplasm.

A series of seven different, commonly used antigen retrieval methods was tested in this work, including pre-treatment omission, enzymatic digestion, heat pre-treatment and heat and pressure pre-treatment. The implementation of heat-based antigen retrieval methods has significantly enhanced the number of antigens that can be detected by immunohistochemistry of formalin-fixed tissues (Shi et al., 1991). Thus, similar to what is frequently observed with other antigens, antigen retrieval omission and enzymatic digestion were less effective than heat-based antigen retrieval procedures for most of the antigens used in the present work. However, the epitope(s) recognized by one of the antibodies (Bak antibody no. 2) showed the opposite behaviour, likely due to increased heat-induced background. The practical significance of this finding is unclear at this point.

Another important aspect that can be addressed with the present system is the optimal incubation condition with the primary antibody. In the present work two different incubation conditions, i.e. 1 h at room temperature and over night at 4 °C, were tested, while the dilution was maintained constant at 1 in 100. Lower antibody dilutions might have revealed more cross-reacting antibodies, however, the suitability of such antibodies for use on tissues would have been questionable. The two incubation conditions used in this study may yield different results depending on the specific antigen/antibody kinetics. The extended incubation at low temperature was effective with bacteria expressing the pro-apoptotic protein Bax, especially

when it was combined with a potent antigen retrieval procedure. It is likely that in this case (and in the case of Bak) the total amount of specific protein expressed in the bacteria was lower than for other proteins due to a killing effect of these pro-apoptotic proteins on bacteria. In this study, however, the labelling intensity was not normalized to the amount of fusion proteins contained in the bacteria. For this purpose, immunohistochemistry of the bacterial pellets using an antibody against GST was attempted, but did not result in labelling of the fusion proteins (not shown). Due to the lack of standardization of protein concentration, antibody titre comparisons in this study are only valid between antibodies against the same Bcl-2 family protein. Ideally, titre assessment using this system could allow selection of antibodies with high affinity for further use on tissues. The antibodies against Mcl-1 and Bcl-x chosen for the second part of the work showed a titre within the range of the majority of the antibodies tested. In conclusion, further investigations should determine how far findings from studies with formalin-fixed, paraffin-embedded bacteria expressing recombinant proteins can be conveyed to IHC on tissues, in particular with regard to antigen retrieval and antibody incubation conditions.

A potential limitation of this test system is constituted by the occurrence of background labelling, defined in this work as a consistent labelling of similar intensity of all bacterial pellets. In some cases, any specific reaction might be covered by the background reaction, thus rendering further investigations necessary for antibodies showing this effect. Background labelling can be derived from reactions either with bacterial proteins (Keller et al., 2007) or with the overexpressed GST moiety of the fusion proteins. In this situation, Western blot analysis is the method of choice, since it allows use of the size of the labelled bands as a discriminative criterion. Western blots performed with three of the antibodies (Mcl-1 no. 1, Noxa no. 1 and 2) showing a variable degree of background labelling with the formalin-fixed bacteria did not result in labelling of any bands. This finding suggests a lack of cross-reactivity of these antibodies with the canine proteins. The reason for the background reaction remains unknown in these particular cases. It may originate from non-specific conformational epitopes.

The aim of the second part of this study was to determine the expression of the anti-apoptotic proteins Mcl-1 and Bcl-x in normal canine tissues and lymphoma samples. For this purpose, immunohistochemical protocols with selected specific cross-reacting antibodies were optimized for use on tissues. The tools used to this end included formalin-fixed, paraffin-embedded UVB-irradiated and non-irradiated cultured canine keratinocytes and

normal skin and lymph node samples. Consistent expression data have been reported for these tissues. Moreover, the investigation of normal lymph nodes is appropriate prior to studying lymphomas. In addition, the expression patterns of the two proteins were studied in an extended series of normal canine tissues. Besides generating expression data potentially useful for future investigations, this assessment provides the possibility to compare the results with available data from other species (Appendix B). Taken together, the results from the present study for the tissues listed above were indicative of a good degree of reliability of the detection protocols used.

In the cell culture system, the optimized immunohistochemical protocol for Mcl-1 detection markedly reduced the amounts of this protein in normal canine keratinocytes after UV-irradiation. This was expected, since this stimulus has been shown to be associated with Mcl-1 protein degradation in this cell type (Nijhawan et al., 2003; Chaturvedi et al., 2005). In the canine skin, the Mcl-1 antibody elicited a diffuse labelling throughout all epidermal layers, except for the stratum corneum and a relative intense labelling of dermal structures. This is in some disagreement with previous reports indicating enhanced labelling of the stratum granulosum within the superficial epidermis and a weak labelling of the adnexa (Krajewski et al., 1995; Fernandez-Figueras et al., 2000). Preincubation experiments indicated that while the major portion of the Mcl-1 labelling observed in the normal skin was likely dependent on antibody moieties directed against canine Mcl-1, a variable fraction of the Mcl-1 polyclonal antibody used might react non-specifically in dermal tissues. This notion is supported by the fact that the bacterial pellet expressing GST-Mcl-1 always was completely negative after IHC with preincubated Mcl-1 antibody, suggesting that the antibody was depleted of all moieties specific for canine Mcl-1. The bacterial pellet used is likely to yield such large amounts of fusion protein that should render residual specific antibodies detectable. Alternatively, since preincubation was done using a GST-Mcl-1 fusion protein, it cannot completely be ruled out that a subset of antibodies was not depleted because of steric hindrance of some Mcl-1 epitopes. In general, the findings of this study suggest that this type of control, as it was performed in this study (i.e. antibody preincubation with the species-specific target antigen), is particularly well-suited when antibodies raised against heterologous antigens are used. Further, the Mcl-1 expression pattern observed in canine lymph nodes and consisting in a relatively strong immunostain of the germinal centre lymphocytes and negligible immunoreactivity of lymphocytes in other regions is in accordance with previous findings (Krajewski et al., 1994; Krajewski et al., 1995). The notion that this labelling was specific is further supported by the complete lack of signal detected in

lymphocytes after antibody preincubation with GST-Mcl-1.

Finally, Mcl-1 expression data obtained from the normal canine tissue arrays were roughly in agreement with previously reported findings summarized in Appendix B (Krajewski et al., 1995), with the difference that labelling intensity appeared to be consistently slightly stronger than in the study cited. In tracheal chondrocytes and endocrine pancreas labelling appeared slightly more intense in the previous report than in the own materials. In contrast, labelling reported in the Human Protein Atlas (HPA, www.proteinatlas.org), a publicly accessible repository containing TMA-based immunohistochemical expression data of endogenous proteins in normal and neoplastic human tissues (www.proteinatlas.org) was markedly less pronounced (Appendix B). Except for results in bone marrow cells, which were slightly more intensely labelled in the HPA than in the present work and in Krajewski's study, the expression data of the HPA were indicative of an overall low degree of sensitivity of the immunohistochemical method. This is supported by the fact that, contrary to the present and Krajewski's study, the epidermis was completely negative in the HPA. It is noticeable that the HPA set of data is based on the very same anti-Mcl-1 antibody used in the present work. Differences in labelling intensity for different cell types could hence reflect actual differences in expression between dogs and humans. Alternatively, differences in the detection protocols can lead to significant differences in the results of immunohistochemical studies. In conclusion, the immunohistochemical protocol for Mcl-1 detection developed in this work appeared to be reliable for the detection of this protein in tissues including lymphatic organs. Antibody preincubation appears to be an useful tool to detect occasional likely non-specific reactions.

In contrast to Mcl-1, Bcl-x signal intensity remained constant in cultured keratinocytes after UV-irradiation. This is compatible with previous reports describing a lack of change in the total protein amount following this stimulus. Translocation of Bcl-x from the cytoplasm to the mitochondria was previously described as an UV-dependent change (Nijhawan et al., 2003). Under optimal conditions, this should manifest as change of the signal distribution from diffuse to punctate, which was not observed in this study. Such changes are likely too subtle to be detected by IHC of formalin-fixed cells. In skin specimens, the Bcl-x antibody elicited variable labelling patterns. Specimens enclosing mucocutaneous transition regions showed enhanced labelling of central epidermal layers, which corresponds to findings in human epidermis in one previous report (Krajewski et al., 1994). In the remaining skin specimens in this study, however, all vital epidermal layers were similarly labelled, which is supported by findings of several other studies on human tissues (Thamboo et al., 2006; Pasmatzi et al.,

2007). Taken together, these observations suggest that the epidermal distribution of Bcl-x may vary depending on the localization. In addition, individual differences in the expression of this protein cannot be completely excluded. In normal lymph nodes, the very weak Bcl-x labelling of germinal centre lymphocytes observed, concomitant to a mostly completely negative immunostaining of circumjacent lymphocytes, is also in accordance to previous studies (Krajewski et al., 1994).

The normal tissue array-derived Mcl-1 and Bcl-x expression data in the present study were roughly comparable to those of previous reports based on human tissues with a few minor differences (Krajewski et al., 1994; Krajewski et al., 1995). In contrast, IHC intensity values reported in the HPA for Bcl-x are, in general, considerably lower than in the present study and in a large number of tissues, such as for instance the skin, labelling was completely lacking. This indicates that the protocol used in the HPA for Bcl-x detection is less sensitive than those used in the present and other published studies (Krajewski et al., 1994). Thus, the differences observed likely derive from the use of different antibodies and of different detection protocols and do not reflect differences in expression between canines and humans. In conclusion, the immunohistochemical protocol for Bcl-x detection developed in this work appears to be reliable for the detection of this protein in tissues including lymphatic organs.

Mcl-1 and Bcl-x expression patterns were determined in a set of normal canine organs and a set of lymphoma samples assembled into tissue microarrays (TMAs). The TMA technique enables a high-throughput immunohistochemical evaluation of tissue specimens (Kononen et al., 1998). It permits simultaneous evaluation of a large number of tissue samples processed under identical conditions and saves reagents, tissue samples and time, which improves efficiency and lowers costs (Milanes-Yearsley et al., 2002). In general, this technique is used to investigate tumour samples. In this study it also facilitated the examination of several normal canine tissues. However, the main disadvantage of this technique is given by the limited amounts of tissues available for each case, which may not be representative of the whole specimen. Varying degrees of agreement have been demonstrated between TMAs and whole tissue sections in validation experiments with human tumour samples (Zu et al., 2005). This major deficiency has become evident in the present study with normal organs. A comprehensive and detailed investigation of all cell types included in the tissues examined was prevented by the small amount of tissue available, the occurrence of non-representative tissue cores and the occasional loss of cores. Nevertheless, to the knowledge of the author, expression data in canine tissues for Mcl-1 and Bcl-x are presented here for the first time and appear comparable to those described in previous human studies. For future investigations,

the use of larger tissue cores, a larger number of cores for each tissue type and a larger number of individuals would be beneficial to further improve the quality of normal tissue data.

This is the first report on the expression of the anti-apoptotic proteins Mcl-1 and Bcl-x in canine lymphomas. The data suggest that most canine lymphomas express these proteins to a certain extent. The generally higher level of labelling for Mcl-1 than for Bcl-x reflects the findings in normal tissues and may reflect, at least in part, a different degree of sensitivity of the respective detection protocols. The data of the present investigation suggest that Mcl-1 is frequently expressed to relatively high levels across various canine lymphoma subtypes and that it is more intensely expressed in B-cell- than in T-cell neoplasms. A requirement for Mcl-1 for both development and maintenance of B- and T-cells has been reported (Opferman et al., 2003). It seems therefore plausible that lymphomas may frequently express this protein. Mcl-1 has been shown to be required for survival in a human B-cell lymphoma line (Michels et al., 2004). In the only study on this protein in dogs known to the author, Mcl-1 expression was detected by RT-PCR in a B-cell leukaemia line, but not in a T-cell lymphoma line (Sano et al., 2004). In human lymphoma, levels of Mcl-1 expression detected by IHC have been shown to vary with a considerable portion of negative cases, which seems to differ from the findings in the present work (Krajewski et al., 1994; Cho-Vega et al., 2004; Wendel et al., 2007). In those studies, Mcl-1 was detected more frequently in high-grade than in low-grade lymphomas (88% vs. 37% in a study by Cho-Vega et al., 2004). For diffuse large B-cell lymphomas, percentages of positives ranging from 73% to 97% of the cases have been reported (Cho-Vega et al., 2004; Wendel et al., 2007). Interestingly, studies of lymphatic leukaemia, based on flow-cytometry or Western blot analysis, tend to report figures near to 100% of the cases positive (Campos et al., 1999; Hogarth and Hall, 1999). It is possible that this is a peculiarity of leukaemias. However, a study has reported 100% positives of human cutaneous T-cell lymphomas analysed using IHC on cryostat sections, a more sensitive antigen detection method than IHC of formalin-fixed tissues (Zhang et al., 2003). Thus, the differences between the present study on canine tissues and previous studies on human tissues might rely on different sensitivities of the respective immunohistochemical protocols. The antibody used in the present work was raised against a stretch of 103 amino acids in the human Mcl-1 protein sequence (www.proteinatlas.org), which is far longer than the immunogens utilised to generate the antibodies used in the human studies cited. Thus, it is conceivable that the Mcl-1 antibody used in this study is more sensitive. Conversely, an enhanced number of antibody moieties, as it is likely to occur in such an antibody, increases

the risk of non-specific reactions.

In contrast to Mcl-1, in the present collective, there were no statistically significant differences between immunophenotypes or lymphoma subtypes for the expression level of Bcl-x. However, compared to the level of expression in non-neoplastic T-cells, the expression of Bcl-x was markedly enhanced in the neoplastic counterparts. The only reference to Bcl-x in canine lymphoid tumours, known to the author, reports its expression in one canine lymphoma and one leukaemia cell line (Sano et al., 2003). Studies in human lymphomas have revealed various frequencies of Bcl-x positivity partly depending on the subtype (Bairey et al., 1999; Agarwal and Naresh, 2002). In some instances, expression of Bcl-x had a negative prognostic impact (Bairey et al., 1999). In the present lymphoma samples, there was no correlation between the expression of Mcl-1 and Bcl-x. This indicates that the mechanisms that lead to overexpression of these proteins may vary and that the significance of these two anti-apoptotic proteins differs between cases. Future studies using further antibodies against the proteins investigated in this work are needed to confirm the data and investigations with antibodies against the further anti-apoptotic members of the family, in particular of Bcl-2, are needed to assess the whole spectrum and the quality of Bcl-2 family-related anti-apoptotic signalling in each individual case. Finally, these methods should be applied prospectively to clinical cases to assess the prognostic significance of the expression of these Bcl-2 family proteins.

# 7. Conclusions

The IHC-based test system using recombinant canine Bcl-2 family proteins expressed in bacteria used in this study is useful to select specific cross-reactive anti-human antibodies. Using this system, several antibodies against the proteins Mcl-1, Bcl-x, Bcl-w, Bak and Bax and potentially suited for immunohistochemistry were selected. A further test system based on cultured canine cells fixed in formalin and embedded in paraffin proved useful to develop immunohistochemical protocols for detection of Mcl-1 and Bcl-x in canine tissues using selected antibodies.

Expression patterns in normal canine tissues were determined for these two proteins using the optimized immunohistochemical protocols. These data over a large number of different canine tissues are novel. They indicate that normal canine tissues express these proteins in a similar way than human tissues. In addition, comparison of these results with data from human studies provided further confidence that the immunohistochemical protocols used are reliable. In this respect, preincubation of the primary Mcl-1 polyclonal antibody canine recombinant Mcl-1 proved to be an important specificity control.

Further application of the optimized immunohistochemical protocols to canine lymphomas revealed that these tumours often prominently express Mcl-1 and, to a lesser extent, Bcl-x. This fact suggests a role for these two anti-apoptotic Bcl-2 family members in canine lymphoma.

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**Appendix A:** IHC for Mcl-1 and Bcl-x in canine lymphomas with indicated mean scoring values (for all cores available) for each of the ninety-three classified lymphomas

| Tumour no. | Immunophenotype | Lymphoma subtype | Mean IHC expression score for indicated protein | | |
|---|---|---|---|---|---|
| | | | Mcl-1 | Mcl-1 corrected° | Bcl-x |
| 1 | B | centroblastic monomorphic | 3.0 | 3.0 | 2.8 |
| 2 | | | 3.0 | 2.8 | 2.0 |
| 3 | | | 4.5 | 4.5 | 3.0 |
| 4 | | | 4.2 | 4.2 | 1.5 |
| 5 | | | 5.0 | 5.0 | 1.5 |
| 6 | | | 4.1 | 3.4 | 2.3 |
| 7 | | | 3.0 | 3.0 | 2.1 |
| 8 | | | 4.5 | 4.5 | 1.5 |
| 9 | | | 4.2 | 4.2 | 3.4 |
| 10 | | | 3.1 | 2.1 | 1.3 |
| 11 | | centroblastic polymorphic | 2.5 | 2.5 | 1.8 |
| 12 | | | 5.0 | 5.0 | 1.8 |
| 13 | | | 3.7 | 3.7 | 2.8 |
| 14 | | | 4.2 | 4.2 | 2.4 |
| 15 | | | 4.0 | 4.0 | 2.8 |
| 16 | | | 3.1 | 3.1 | 1.5 |
| 17 | | | 5.0 | 5.0 | 4.1 |
| 18 | | | 3.0 | 2.0 | 1.6 |
| 19 | | | 3.0 | 3.0 | 1.1 |
| 20 | | | 2.8 | 2.8 | 3.9 |
| 21 | | | 1.0 | 1.0 | 4.3 |
| 22 | | | 2.5 | 2.5 | 1.4 |
| 23 | | | 4.0 | 2.8 | 1.3 |
| 24 | | | 4.5 | 3.6 | 1.4 |
| 25 | | | 1.5 | 1.5 | 4.0 |
| 26 | | | 1.8 | 1.3 | 2.8 |
| 27 | | marginal zone | 3.1 | 3.1 | 2.5 |
| 28 | | | 2.8 | 2.3 | 1.5 |
| 29 | | | 2.7 | 1.0 | 2.5 |
| 30 | | | 3.9 | 3.9 | 2.8 |
| 31 | | | 2.2 | 2.2 | 3.6 |
| 32 | | | 3.0 | 3.0 | 2.4 |
| 33 | | | 3.0 | 4.0 | n.a. |
| 34 | | | 3.6 | 3.6 | 2.3 |
| 35 | | | 2.5 | 2.5 | n.a. |
| 36 | | | 2.4 | 1.7 | 1.2 |
| 37 | | | 3.8 | 3.3 | 1.0 |
| 38 | | lymphoplasmacytic | 5.0 | 5.0 | 0.7 |
| 39 | | | 3.1 | 3.4 | 2.8 |
| 40 | | Burkitt | 5.0 | 5.0 | 2.8 |
| 41 | | | 2.4 | 1.9 | n.a. |
| 43 | | immunoblastic | 5.0 | 4.8 | 3.8 |
| 42 | | plasmablastic | n.a. | n.a. | 4.0 |
| 44 | | diffuse large | 5.0 | 5.0 | 2.8 |
| 45 | | follicular | 4.0 | 4.0 | 3.2 |
| 46 | | small B-cell | 4.0 | 4.0 | 3.3 |

| | | | | | |
|---|---|---|---|---|---|
| 47 | B | not classifiable | 4.0 | 3.5 | 4.3 |
| 48 | | | 4.5 | 4.3 | 2.8 |
| 49 | | | n.a. | n.a. | 2.5 |
| 50 | | | 5.0 | 4.3 | 1.8 |
| 51 | | | 2.3 | 2.3 | 2.0 |
| 52 | T | pleomorphic (various sizes) | 2.8 | 2.8 | 1.3 |
| 53 | | | 4.2 | 4.2 | 2.8 |
| 54 | | | 2.5 | 2.4 | 3.8 |
| 55 | | | 2.4 | 2.4 | 2.1 |
| 56 | | | 1.9 | 1.9 | 1.0 |
| 57 | | | 4.9 | 4.9 | 2.8 |
| 58 | | | 1.4 | 1.5 | 2.5 |
| 59 | | | 3.3 | 2.8 | 1.3 |
| 60 | | | 2.0 | 1.5 | 4.0 |
| 61 | | | 2.8 | 2.0 | 2.5 |
| 62 | | | 1.7 | 1.3 | 2.3 |
| 63 | | | 2.4 | 1.9 | 3.2 |
| 64 | | | 2.0 | 2.0 | 2.0 |
| 65 | | | 2.3 | 2.3 | 1.9 |
| 66 | | | 1.8 | 1.8 | 1.5 |
| 67 | | large granular lymphoma | 5.0 | 4.1 | n.a. |
| 68 | | | na | na | 1.0 |
| 69 | | | 4.9 | 4.9 | 3.0 |
| 70 | | | 4.8 | 4.8 | 1.5 |
| 71 | | | 2.3 | 2.3 | 3.0 |
| 72 | | lymphoblastic | 2.0 | 2.0 | 3.5 |
| 73 | | | 2.1 | 2.0 | n.a. |
| 74 | | plasmacytoid | 2.2 | 1.8 | 0.5 |
| 75 | | cytotoxic | 4.0 | 4.0 | 3.7 |
| 76 | | small clear cell type | 2.3 | 3.0 | 2.6 |
| 77 | | mycosis fungoides | 3.0 | 1.8 | 3.5 |
| 78 | | not classifiable | 3.1 | 3.1 | 2.3 |
| 79 | | | 2.8 | 2.8 | 4.0 |
| 80 | | | 2.5 | 2.5 | 1.8 |
| 81 | | | 2.1 | 2.1 | 0.8 |
| 82 | | | 1.9 | 1.9 | 1.3 |
| 83 | DN | high-grade, medium sized | 3.7 | 3.8 | 4.4 |
| 84 | | | 4.5 | 0.6 | 4.5 |
| 85 | | | 3.5 | 3.5 | 2.5 |
| 86 | | | 5.0 | 4.5 | 1.6 |
| 87 | | | 2.5 | 2.3 | 1.0 |
| 88 | | plasmacytoid | 1.7 | 1.2 | 3.2 |
| 89 | | immunoblastic | 1.5 | 0.7 | 3.5 |
| 90 | | mycosis fungoides | 3.1 | 3.1 | 3.7 |
| 91 | | not classifiable | 3.0 | 3.0 | 4.4 |
| 92 | | | n.a. | n.a. | n.a. |
| 93 | | | 5.0 | 5.0 | 2.3 |

° Mcl-1 corrected=score value after IHC with antibody Mcl-1 no. 2 minus score value after IHC with preincubated Mcl-1 antibody
n.a.=not available because of lack of representative tissue cores

**Appendix B:** Comparison of IHC expression data of Mcl-1 and Bcl-x in normal canine tissues with published human data

| Tissue/organ | Cell type | IHC labelling intensity for indicated protein in indicated study | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mcl-1 | | | Bcl-x | | |
| | | Present study | Krajewski° | Proteinatlas* | Present study | Krajewski° | Proteinatlas* |
| **Skin** | | | | | | | |
| Epidermis | Basal cell layer | 2-3 | 1-2 | 0 | 1/2-3 | 0-1 | 0 |
| | Spinous layer | 2-3 | 1-2 | 0 | 2-3 | 1-3 | 0 |
| | Granular layer | 2-3 | 2-4 | 0 | 3 | 2-4 | 0 |
| | Cornified layer | 0 | 0-1 | 0 | 0 | 0-1 | 0 |
| Dermis | Sweat glands | 5 | 1-2 | | 1-2 | 1-2 | |
| | Sebaceous glands | 3-4 | 0-1 | | 3 | n.a. | |
| | Hair follicles | 3-4 | 0-1 | | 2-3 | n.a. | |
| | Endothelium | 3 | n.a. | 1 (adnexal cells) | 3 | n.a. | 0 (adnexal cells) |
| | Muscles | 1-2 | n.a. | | 2 | n.a. | |
| | Fibrocytes | 5 | 0-1 | | 0/2 | 0-2 | |
| | Peripheral nerves | 3 | 0 | | 0-1 | n.a. | |
| **Digestive** | | | | | | | |
| Salivary glands | Serous acini | 5 | n.a. | 0 | 0 | n.a. | 0 |
| | Mucous acini | 0 | n.a. | 0 | 0 | n.a. | 0 |
| | Excretory duct | 3/5 | n.a. | 0 | 2 | n.a. | 0 |
| Stomach | Parietal cells | 4 | 1-2 | 2 (glandular cells) | 3-4 | 1 | 0-1 (glandular cells) |
| | Chief cells | 4 | 0-1 | | 0 | 1 | |
| Intestine | Enterocytes | 4 | 2-4 | 2 | 1/4 | 1-3 | 0 |
| Liver | Hepatocytes | 2/4 | 0 | 2 | 2/4 | 1-2 | 0 |
| Pancreas | Exocrine cells | 0/5 | 0-1 | 0 | 1 | 0-1 | 0 |
| | Islets of Langerhans | 1-2 | n.a. | n.a. | 2-3 | 0 | n.a. |
| **Respiratory** | | | | | | | |
| Trachea | Epithelium | 2 | 1-4 | n.a. | 2-3 | 2-3 | n.a. |
| | Chondrocytes | 0 | 2-4 | n.a. | 0 | n.a. | n.a. |
| Bronchi | Epithelium | 1-2 | 1-2 | 1 | 1 | 1-2 | 0 |
| Alveoli | Pneumocytes | 0/4 | 0 | 0 | 0 | n.a. | 0 |
| | Alveolar macrophages | 4-5 | 3-4 | 2 | 2/4 | n.a. | 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Cardiovascular** | | | | | | | |
| Heart | Myocardium | 1-2 | 1-3 | 0 | 1-2 | 2-3 | 0 |
| **Reproductive** | | | | | | | |
| Ovary | Germinal epithelium | 2 | 1-2 | n.a. | 3 | n.a. | n.a. |
| | Corpus luteum | 4 | 1-2 | n.a. | 3 | n.a. | n.a. |
| Uterus | Endometrium | 3-4 | 0-1 | 1 | 2-3 | 3-4 | 0 |
| Vagina | Epithelium | 2-3 | 2-3 | 1 | 0/2 | n.a. | 0 |
| Mammary gland | Epithelium | 4 | 1-2 | 1 | 1-2 | 3-4 | 0 |
| Prostate | Epithelium | 5 | 1-3 | 2 | 3-4 | 1-2 | 1 |
| Testis | Spermatogonia | 0/5 | 0 | 1 (cells in seminiferus ducts) | 1-2 | 1 | 0 (cells in seminiferus ducts) |
| | Spermatocytes | 0/3 | 0-1 | | 1-2 | 0 | |
| | Spermatids | 0/3 | 0-1 | | 1-2 | 1 | |
| | Sertoli cells | 1/3 | 0 | | 1-2 | n.a. | |
| | Leydig cells | 5 | 2-4 | 1 | 3-4 | n.a. | 0 |
| **Musculoskeletal** | | | | | | | |
| Skeletal muscle | Myotubules | 1 | 1-2 | 1 | 1/4 | 1-2 | 0 |
| **Hematopoietic/lymphoid** | | | | | | | |
| Spleen | White pulp lymphocytes | 2 | 0-4 | 1 | 0 | 0 | 0 |
| | Red pulp cells | n.a. | 0-1 | 2 | 1-2 | 0 [1-3]$^{\dagger}$ | 0 |
| Tonsil/lymph node | Germinal centre lymphocytes | 3-4 | 1-4 | | 1-2 | 0 [2]$^{\ddagger}$ | |
| | Interfollicular lymphocytes | 2-3 | 2-4 | 2, 3$^{\Delta}$ | 0-1 | 0 [4]$^{\dagger}$ | 0, 1$^{\circ\circ}$ |
| | Mantle lymphocytes | 0/2 | 0-1 | | 0-1 | 0 | |
| Thymus | Cortical thymocytes | 0-1 | 0-4 | n.a. | 0 | 2 | n.a. |
| | Medullary thymocytes | 0-1 | 0-1 | n.a. | 0 | 0-1 | n.a. |
| | Epithelioreticular cells | 2-3 | 3-4 | n.a. | 2-3 | 0-1 | n.a. |
| Bone Marrow | Myeloid precursors | 1-2 | 0-2 | 3 | 1-2 | 0-3 | 1 |
| | Erythrocyte precursors | 1-2 | 1-2 | bone marrow poietic cells | 1-2 | 2-4 | bone marrow poietic cells |
| | Megakaryocytes | 1 | 1 | | 1 | 1-2 | |
| **Urinary** | | | | | | | |
| Kidney | Glomeruli | 1 | 0 | 0 | 0 | 0 | 0 |
| | Proximal tubules | 3-4 | 0-1 | 2 | 2-3 | 0-1 | 0 |
| | Distal tubules | 4-5 | 1-3 | 2 | 3-4 | 1-2 | 0 |
| Bladder | Epithelium | 3-4 | 0-4 | n.a. | 2/4 | 2 | n.a. |
| **Endocrine system** | | | | | | | |
| Thyroid | Follicular epithelium | 5 | 0-1 | 1 | 1-2 | 0 | 0 |
| **Central nervous system** | | | | | | | |
| Brain | | | | | | | |
| Cortex | Large neurons | 4-5 | 0-1 | 0 | 1-2 | 0-2 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Neuropil | 0/1 | 1 | 0 | 2-3 | n.a. | 0 |
| | Neuroglia | 0/5 | 0 | 0 | 0/5 | 0 | 0 |
| | Axons in white matter | 2/4 | 0-1 | n.a. | 1/3 | n.a. | n.a. |
| Cerebellum | Purkinje cells | 4-5 | n.a. | 0 | 0/1 | n.a. | 0 |
| | Neurons | 4-5 | n.a. | n.a. | 1-2 | n.a. | n.a. |
| Spinal cord | Neurons | 4-5 | 0-1 | n.a. | 1-2 | 0-2 | n.a. |
| | Neuroglia | 0/5 | 0 | n.a. | 0/5 | 0 | n.a. |
| | Meylin fibers | 2/4 | 0 | n.a. | 1/3 | 0 | n.a. |
| | Neuropil | 0/1 | 1-2 | n.a. | 2-3 | n.a. | n.a. |
| | Ependyma | 4-5 | n.a. | n.a. | 3-4 | n.a. | n.a. |

n.a.=not available

° Data for human tissue extracted from previous publications (Krajewski et al., 1994; Krajewski et al., 1995). Immunostaining results were scored on a five-point scale: 0=no immunoreactivity, 1=weak immunostain, 2=moderate, 3=strong, 4=intense immunoreactivity

* Data from human tissues extracted from Human Protein Atlas (www.proteinatlas.org). Immunostaining results were scored on a four-point scale: 0=negative, 1=weak, 2=moderate, 3=strong immunoreactivity

† Plasma cells and scattered activated lymphocytes were Bcl-x positive.

Δ 2: Lymphoid cells outside germinal centre; 3: Lymphoid cells in germinal centres

°° 0: lymphoid cells in and outside germinal centres of lymph nodes and outside germinal centres of the tonsil; 1: lymphoid cells in germinal centres of the tonsil